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(54) Title: IMPROVED NOVEL ASSAY FOR SCREENING COMPOUNDS CAPABLE OF INDUCING DRUG METABOLIZING ENZYMES

(57) Abstract: Described are methods for screening inducers of genes involved in drug metabolization. In particular, an in vitro cell based assay is provided with cells derived from the cell line LS174T. Described are also methods for identifying and obtaining drugs for therapy of disorders related to the expression of the cytochrome P450 (CYP), gene family. Furthermore, kits suitable for such methods are provided.

Title of the invention

Improved novel assay for screening compounds capable of inducing drug metabolizing enzymes

Field of the invention

The present invention relates generally to means and methods of screening substances that are capable of inducing enzymes involved in drug metabolism. In particular, the present invention relates to methods for identifying and obtaining drug candidates for therapy of disorders related to the expression of a drug metabolizing enzyme or drug transporter encoding gene comprising the use of a cell derived from the cell line LS-174T (ATCC No. CL-188). The present invention furthermore provides kits that are particularly useful for carrying out such a method. The methods and diagnostic kits of this invention yield information concerning the potential inducibility of genes encoding metabolizing enzymes such as cytochromes P450 (CYP) and in particular cytochrome P3A4 by drug candidates. This information may be utilized to design antagonists to compounds found to be inducers or inhibitors of CYP transcription and in active drug design.

The invention described herein draws on previously published work and, at times, on pending patent applications. By way of example, such work consists of scientific papers, abstracts, or issued patents, and published patent applications. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated herein by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

Background of the invention

Members of the cytochrome P-450 (CYP) family of hemoproteins metabolize a wide variety of endogenous substrates such as steroid hormones, and of xenobiotics including carcinogens, toxins and drugs (Daly, Toxicol Lett. 102-103 (1998), 143-147, Touw, Drug Metabol. Drug Interact 14 (1997), 55-82). Of the human CYP proteins, those of the CYP3A subfamily are of a major importance, since collectively, they are by far the most abundant of all the human CYP isoforms. Moreover, their substrate specificity is extremely broad; accordingly, many structurally diverse compounds are, exclusively or to some extent, substrates for CYP3A proteins. Based on the data available it is generally assumed that all CYP3A isoforms have similar substrate spectra; however, limited studies indicate the possibility of differences (Thummel, Annu. Rev. Pharmacol. Toxicol. 38 (1998), 398-430). All CYP3A isoforms are localized in organs of particular importance to drug disposition (gastrointestinal tract, kidney and liver). Cytochrome P450 (CYP) 3A4 is the most prominent CYP in human liver and intestine. It accounts for 25-30 % of total CYP in liver (Shimada et al., 1994) and for an even larger portion of total CYP in intestine (De Waziers et al., 1990 / Watkins et al., 1987). CYP3A4 metabolizes more than 50 % of the clinically used drugs (Wacher et al., 1995). Its activity and expression are varying 10 to 30 fold between individuals (Lown et al., 1994 / Guengerich, 1995 / Paine et al., 1997), so that the level of expression of CYP3A4 can be an important parameter for the therapeutic success of drug treatment. Furthermore this expression can be induced by a variety of drugs and endogenous compounds like rifampicin, barbiturates and glucocorticoids. Induction produces many clinically relevant drug interactions. With respect to drug development, it is therefore important to determine, whether a compound is an inducer of CYP3A4 or not. Up to now the potency to induce CYP3A4 can only be tested by treating cultured primary human hepatocytes with the compounds. But using primary human hepatocytes has some disadvantages. First availability is not unlimited. Secondly the material is coming from different individuals and therefore the system can not be standardized. Thirdly interindividual variability in the induction of CYP3A4 in human hepatocytes has frequently been observed. In one study induction of CYP3A4 expression by rifampicin, one of the strongest known

inducers, varied between 1.5 and 8.5 fold (Kocarek et al., 1995), in another one induction varied between 3.7 and 24 fold (Daujat et al., 1991). High interindividual variability of induction has also been reported for triacetyloleandomycin, dexamethasone, phenobarbital and clotrimazole (Daujat et al., 1991). But even more than quantitative effects have been reported. For example PCN is able to induce CYP3A4 expression in some individuals and not in others (Kocarek et al., 1995). In another study rifampicin is inducing CYP3A4 while phenobarbital is not inducing in the same primary hepatocytes (Morel et al., 1990). These examples demonstrate the need for a well characterized model system for studying CYP3A4 induction. Establishing such a model system requires understanding the molecular mechanisms of induction. But these mechanisms have not yet been completely resolved. Evidence for a transcriptional mechanism of induction mainly comes from investigations of the glucocorticoid induction of CYP3A genes in rat. Dexamethasone treatment elevates the level of mRNA in rat liver (Wrighton et al., 1985 / Komori and Oda, 1994) and stimulates de novo transcription in nuclear run on experiments (Simmons et al., 1987 / Telhada et al., 1992). Sequence comparison of the promoter regions of CYP3A genes of different species identified a conserved region which seems to be essential for the induction by dexamethasone and rifampicin in cultured liver cells (Barwick et al., 1996). Regarding transcriptional activators, the glucocorticoid receptor (GR) might be involved in induction, because it has been shown recently that rifampicin is a high affinity ligand for GR (Calleja et al., 1998). Activation of GR by rifampicin is specific for some cell types only, like hepatocytes and hepatoma cells, but is not occurring in others, like kidney and pituitary cells (Calleja et al., 1998 / Ray et al., 1998). On the other hand lack of binding of GR to the elements in the promoter of rat CYP3A genes which mediate dexamethasone induction has been demonstrated (Huss et al., 1996). Recently it has been shown that a newly identified nuclear hormone receptor, pregnane X receptor (PXR) binds to DNA elements that are also present in the identified promoter regions mediating induction of rat and human CYP3A genes and is activated by many compounds inducing CYP3A (Bertilsson et al., 1998 / Blumberg et al., 1998 / Kliewer et al., 1998 / Lehmann et al., 1998). But these studies have been done exclusively with artificial promoter constructs containing isolated binding sites and not in the context of the CYP3A4 promoter

and also in cell systems where CYP3A4 is normally not expressed nor inducible. Up to now it has not been shown that the CYP3A4 promoter is really regulated by one of the proposed mechanisms involving GR or PXR. Furthermore the regulation and induction of CYP3A genes has been studied merely in liver-derived model systems, like primary hepatocytes or hepatoma cell lines. But evidence is also existing that the expression of CYP3A genes is regulated in a tissue-specific way. No correlation can be found between CYP3A4 expression in liver and intestine of the same individual (Lown et al., 1994). Induction of rat CYP3A by dexamethasone reveals to be tissue-specific. Dexamethasone induces CYP3A in liver, stomach and small intestine (jejunum), but not in oesophagus and colon (Kolars et al., 1992). Recently, refinement of an in vitro cell model for cytochrome P450 induction in primary hepatocytes with different culture conditions has been described by Silva et al. in Drug Metab. Dispos. 26 (1998), 490-496. Likewise, Kostrubsky et al. reported on the use of human hepatocyte cultures to study the induction of cytochrome P450 (Kostrubsky, Drug Metab. Dispos. 27 (1999), 887-894). The authors used primary human hepatocyte cultures to investigate induction of CYP by xenobiotics. Induction by taxol and rifampicin is achieved, but the fold increase was different between cultures of separate donors. The authors showed that this is in part explained by different basal levels of CYP3A4. The cultures having the lowest basal CYP3A4 expression showed the greatest fold-increases. They conclude that the interindividual variability in induction depends on the variable basal expression. In contrast, the maximal extent of CYP3A4 expression induced was similar in the six cultures studied.

Because drugs that induce CYP and/or other genes involved in drug metabolism and drug transport may affect the metabolism of coadministered drugs as well as their own metabolism, it would be highly desirable to have a reliable and economic assay system to determine whether the drug under development has the capacity to induce specific forms of such genes, for example CYP. In view of the short comings of the prior art, there is still a need of such a system today.

Summary of the inv ntion

Applicant has fulfilled this need by providing an in vitro diagnostic kit and assay method which identify and characterize potential inducers of genes involved in drug metabolism in an animal cell. These kits and methods employ cells derived from a particular cell line developed from surgically-derived epithelial cells of human colonic adenocarcinoma tissue and the native promoters of genes involved in drug metabolism, preferably cytochrome P450 (CYP), and measure the level of the activity of a protein which is encoded by a gene which is operatively linked thereto.

In particular, the present invention is based on the finding of novel, so far unknown properties of cells derived from the cell line LS-174T (ATCC No. CL-188). Based upon the knowledge of these novel properties diagnostic tests and reagents for such tests were designed for the detection of specific inducers of genes involved in drug metabolization in the cell. It may also be useful in the determination of inhibitors of gene expression, i.e. compounds that antagonize the action of inducers. Also provided are diagnostic kits comprising cell lines, media, and reagents for use in one of the methods. Various other objects and advantages of the present invention will become apparent from the Detailed Description of the Invention.

Brief Description of the Drawings

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows induction of CYP3A4 expression by rifampicin.
(A) Time course of CYP3A4 induction: Northern Blot analysis of polyadenylated RNA of LS174T and LS180 cells untreated, treated with 10 μM rifampicin for the indicated time periods in days (1d, 2d, 3d) or treated with 0.1% DMSO (vehicle) for

3 days. The blots were sequentially hybridized with probes specific for the genes indicated.

(B) Concentration dependent induction of CYP3A4: Northern Blot analysis of polyadenylated RNA of LS174T cells treated for 48 hours with 0.1% DMSO (vehicle) or with the indicated concentrations of rifampicin. The blot was sequentially hybridized with probes specific for the genes indicated.

Figure 2 shows induction of the CYP3A4 promoter by rifampicin.

Reporter gene constructs containing unidirectional deletions of the CYP3A4 promoter region are shown schematically on the left. Numbers indicate positions (in bp) relative to the transcriptional start site (arrow). The results of transfection experiments are shown on the right: LS174T cells were transfected with the appropriate reporter gene and the β-galactosidase reference plasmid pCMVβ and treated with 10 μM rifampicin or 0.1% DMSO (solvent control) for 42 hours. Then the cells were harvested and analyzed for luciferase and β-galactosidase activity. The columns show the average induction factor of the reporter genes by rifampicin treatment. The activity of each reporter gene in the absence of rifampicin was designated as 1. Thin lines show standard deviations.

Figure 3 shows mutation analysis of regions of the CYP3A4 promoter possibly involved in rifampicin induction.

CYP3A4 promoter reporter genes are shown schematically on the left. The rectangle denotes a region with several imperfect Glucocorticoid receptor element (GRE) half site, which is deleted in Δ250-210. The circle denotes a palindromic $AG^{G}/_{T}TCA$ repeat motif (ER6: 5'-TGAACTCAAAGGAGGTCA-3' (SEQ ID NO: 6)) for nuclear receptors. mER6 denotes a mutated ER6 element (5'-TGTTCTCAAAGGAGAACA-3' (SEQ ID NO: 7)). The results of transfection experiments are shown on the right: LS174T cells were transfected with the appropriate reporter gene and pCMVβ and treated for 42 hours with 10 μM rifampicin or 0.1% DMSO. Then cells were harvested and analyzed for luciferase and β-galactosidase activity as described in figure 2.

Figure 4 shows involvement of the Pregnane X receptor (PXR) in rifampicin induction.

(A) Expression of PXR in human hepatoma and intestinal cell lines: Northern blot analysis of polyadenylated RNA of the indicated human cell lines. The last 3 lanes show a cell density dependent differentiation of Caco-2 TC7 cells. 1: subconfluent. 2: confluent. 3: 15 days post-confluent. The blot was sequentially hybridized with probes specific for the genes indicated. Two different exposures of the hybridization with the PXR specific probe are shown.

(B) Recombinant PXR protein expression: Western Blot analysis of total cellular protein of COS-1 cells transiently transfected with an eukaryotic expression plasmid for human PXR (+) or with the empty expression vector (-). PXR protein expression was analyzed using a PXR-specific antibody. Molecular weight markers (in kilodaltons) are shown on the left. The activity of the co-transfected pCMVβ plasmid was determined in aliquots of the transfected cells to ensure that the transfection efficiencies were similar.

(C) Rifampicin-induced activation of the CYP3A4 promoter by PXR: LS174T cells were co-transfected with the CYP3A4 promoter reporter gene containing the region up to –1105 bp, pCMVβ and an expression plasmid for human PXR (+) or empty expression vector (-) and treated for 42 hours with 10 μM rifampicin (+) or 0.1% DMSO (-). Then the cells were harvested and analyzed for luciferase and β-galactosidase activity. The columns show the average induction factor. The activity in the absence of rifampicin and exogenous PXR protein was designated as 1. Thin lines show standard deviations.

Figure 5 shows induction profile of endogenous CYP3A4 expression.

Northern Blot analysis of polyadenylated RNA of LS174T treated for 2 days with 0.1% DMSO, 50 μM coumestrol, 50 μM carbamazepine or 10 μM of the other compounds. The blot was hybridized with a probe specific for CYP3A4. Equal loading of RNA was confirmed by inspection of the blot with UV light (UV shadowing).

Figur 6 shows induction profile of CYP3A4 promoter by PXR.
LS174T cells were co-transfected with the CYP3A4 promoter reporter gene containing the region up to –1105bp, pCMVβ and an expression plasmid for human PXR and treated for 42 hours with 0.1% DMSO, 50 μM carbamazepine or 10 μM of the other compounds. Then the cells were harvested and analyzed for luciferase and β-galactosidase activity. The columns show the average induction factor. The activity of DMSO-treated cells was designated as 1. Thin lines show standard deviations.

Figure 7 shows triplicates resulting from induction with 10 μM Rifampicin.
The absolute luciferase activity is shown without and with compensation by β-galactosidase acitivity.

Figure 8 shows compensation for toxic effects for RU486 induction.
The cytotoxic and stress effects occurring after treatment of the cells with 30 μM RU486 can be normalized by determining the β-galactosidase activity.

Figure 9 shows the influence of the solvent on luciferase activity in inductive assays. Non-inducers such as Doxorubicin or Staurosporin have similar to PBS and 0.01% DMSO no inducing effect on reporter gene luciferase, while 3% DMSO induces significantly luciferase activity.

Detailed Description of the Invention

The above and various other objects and advantages of the present invention are achieved by (a) providing cells that contain substantially all prerequisites that allow specific induction of genes involved in drug metabolization, (b) constructing recombinant vectors containing promoters and/or response elements of such genes and in particular CYP3A4, so that cells when infected or transfected with said recombinant vectors are efficiently responsive to compounds that are capable of inducing the corresponding gene in vivo; and (c) providing means which are

suitable for compensation of sources of false negative and/or false postive results. Accordingly, the invention relates to a method of determinig whether a compound is an inducer of a drug metabolizing enzyme comprising the steps of:

(a) culturing cells of the cell line LS-174T (ATCC No. CI-188) or cells derived therefrom in a nutrient medium essentially free of steroids and capable of supporting the growth of said cells, wherein said cells comprise at least one gene encoding a protein capable of regulating the expression of said drug metabolizing enzyme and a first recombinant DNA molecule which comprises a heterologous promoter operatively linked to a gene encoding a first detectable product and a second recombinant DNA molecule which comprises nucleotides –7830 to +51 or at least one repeat of nucleotides –7830 to –7209 and nucleotides –363 to +51 of the CYP3A4 gene (Accession number: AF185589) operatively linked to a gene encoding a second detectable product;

(b) incubating said cells with a first amount of a compound or a sample comprising a plurality of compounds suspected to be an inducer under conditions which permit expression of said first and said second detectable product;

(c) quantifying said first and said second detectable product;

(d) determining the ratio of the amount of said second and said first detectable product; and

(e) comparing the ratio determined in step (d) with the ratio obtained in step (d) of a corresponding assay with a control substance, wherein an increased ratio is indicative for an inducer.

In accordance with the present invention, the mechanisms of Cytochrome P450 induction were examined by way of example through intestinal CYP3A4 induction by the model inducer rifampicin because of the growing evidence of an important role of the intestine for first pass metabolismus during the last years and because rifampicin preferentially induces the prehepatic and not the hepatic metabolism of drugs like verapamil (Fromm et al., 1996 / Fromm et al., 1998). Fortunately, it turned out that the human colon carcinoma cell line LS174T is a suitable model system for studying the molecular mechanisms of intestinal CYP3A4 induction. The

results also demonstrate that the profile of expression is very akin to the pattern of induction/expression of the endogenous gene and that the cell line LS174T can thus be used for developing a model system to screen for inducers and inhibitors of genes involved in drug metabolization or transport.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications and patent documents referenced in this application are incorporated herein by reference.

The term "determining whether a compound is an inducer" comprises identifying a compound as a inducer and optionally elucidating its chemical structure.

The term "cells derived from cell line LS-174T" means cells that have the essential characteristics of cells of cell line LS-174T (ATCC No. CL-188), for example the same karyotyp. Such cells may also be obtained from the parent cell line LS-180 (ATCC No. CL-187) as described in US-A-4,228,236, the disclosure of which is incorporated herein. Further characteristics of such cells which also distinguish them from, e.g., cell line LS-180 are population doubling time, or release of carcinoembryonic antigen (CEA). Preferably, cells directly originated from cell line LS-174T are used in the methods and kits of the present invention. Preferably, the cells retain the inducing capabilities of LS-174T cells, in particular with respect to rifampicin.

The term "response element" as herein after refers to a region of a nucleic acid molecule, usually, from a regulatory region of a gene such as a promoter or an enhancer, that is capable of specifically binding to a binding protein, such as an activator molecule, for activation of transcription or for allowing the elongation of a RNA transcript, or a repressor molecule, for inhibition of transcription. Furthermore, there is evidence that small molecules such as the compounds of the present invention may also bind to said response elements or to response elements similar thereto. Some response elements are known in the art. Selection of a response element that is suitable for use herein is within the capability of one skilled in the art.

The term "regulates," in the context of transcription or gene expression, denotes both positive and negative regulation. Positive regulation is exemplified by induction. Negative regulation is exemplified by repression.

A compound that induces the action of a drug metabolizing enzyme, either induces or enhances its enzymatic activity or its expression. The term "compound" in a method of the invention includes a single substance or a plurality of substances which may or may not be identical. Said compound(s) may be chemically synthesized or produced via microbial fermentation but can also be comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be useful as a modulator. The plurality of compounds may be, e.g., added to the culture medium or injected into the cell. If a sample containing (a) compound(s) is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound, in question or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. It can then be determined whether said sample or compound displays the desired properties, for example, by the methods described herein or in the literature. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical. The methods of the present invention can be easily performed and designed by the person skilled in the art, for example in accordance with other cell based assays described in the prior art or by using and modifying the methods as described herein. Furthermore, the person skilled in the art will readily recognize which further compounds and/or enzymes may be used in order to perform the methods of the invention, for example, enzymes, if necessary, that convert a certain compound into the precursor which in turn represents a substrate for the protein. Such adaptation of the method of the invention is well within the skill of the person skilled in the art and can be performed without undue experimentation.

Particularly when employed in the above described methods, can be used for pharmacological and toxicological studies of the metabolism of drugs. Preferred drugs to be tested in accordance with the methods of the present invention comprise those described above and include, but are not limited to nifedipine, erythromycin, troleandomycin, quinidine, cyclosporin A, 17 α-ethynylestradiol, lidocaine, diltiazem, dexamethasone, RU486, rifampicin, omeprazole, clotrimazole and derivatives thereof; see also supra.

Compounds which can be used in accordance with the present invention include peptides, proteins, nucleic acids, antibodies, small organic compounds, ligands, peptidomimetics, PNAs and the like. Said compounds can also be functional derivatives or analogues of known drugs such as from those described above. Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art or as described. Furthermore, peptide mimetics and/or computer aided design of appropriate drug derivatives and analogues can be used, for example, according to the methods described below. Such analogs comprise molecules having as the basis structure of known modulators. In summary, the present invention provides methods and kits for identifying and obtaining compounds which can be used in specific doses for the treatment of specific forms of diseases, e.g., cancer the chemotherapy of which is complicated by altered expression of a drug metabolizing enzyme gene often resulting in an altered activity or level of drug metabolization or sensitive phenotypes.

The drug metabolizing enzymes, drug transporters and proteins regulating the expression of the same such as orphan nuclear receptors themselves have evolved as an adaptive response system against the toxic challenge of environmental chemicals. As part of this response, exposure to a particular chemical generally induces the expression of specific proteins active in the metabolism of that chemical, often in a tissue specific manner. The basal level of expression is extremely low or negligible and the induction of gene expression can

be significant. Accordingly, the present invention employs a recombinant DNA molecule comprising a detectable product, usually a protein operably linked to an expression control sequence of a gene regulated by drugs or a xenobiotic compound, wherein the expression control sequence comprises at least one promoter of the gene or a response element thereof, and wherein the basal expression of the gene of the recombinant DNA molecule is significantly reduced whilst allowing the induction of expression of the detectable product in a manner similar to which the gene is regulated in vivo. This could preferably be the CYP3A4 promoter as shown in the examples or any p450 type promoter of any gene encoding a drug metabolizing enzyme or any gene encoding a drug transporter, said genes known to be regulated by chemicals. The promoter or response element may be naturally occurring or genetically engineered. A genetically engineered promoter may comprise at least one up to a non-limited number of response elements. The best characterized promoter of such enzymes is the regulation through a promoter termed the xenobiotic regulatory element (XRE). Preferably, a promoter is to be found in a fragment comprising nucleotides –363 to +51 of the CYP3A4 gene (Accession No: AF185589) while the response elements could be found in a fragment comprising nucleotides –7830 to –7209 of the CYP3A4 gene. Thus, promoter and response elements may be located in a fragment comprising nucleotides –7830 to +51 of the CYP3A4 gene. The indicated positions, however, may vary due to individual differences. Moreover, extended fragments of the above described fragments may comprise further response elements.

Within the scope of the invention are also genetically engineered or artificially synthesized DNA molecules which may comprise at least one of the above response elements.

The above described nucleotide positions refer to a reference sequence for CYP3A4 such as Accession No: AF185589). However, as described above, said positions may vary individually as a result of nucleotide insertions and/or deletions.

How to construct the above defined recombinant DNA molecule is well known to the person skilled in the art and described in the art. For example, WO97/23635 describes recombinant DNA molecules wherein a protein encoding sequence is operably linked to an expression control sequence of gene regulated by drugs or a

xenobiotic compound, such as a drug metabolizing enzyme, wherein the expression control sequence comprises at least one promoter region and at last one repressor region wherein the at least one repressor region significantly reduces basal expression at least in a tissue where the protein is required to be expressed whilst allowing the induction of expression of the protein encoding sequence in a manner similar to which the gene is regulated in vivo.

Preferred control substances comprise solvents which are suitable to dissolve the compounds suspected to be inducers, such as DMSO, ethanol or methanol.

Preferably, said corresponding assay with a control substance is performed simultaneously with the assay to determine whether said compound suspected to be a inducer is a inducer. Usually, said assays are performed in microtiter plates to ensure equal conditions and to increase reliability. Preferred microtiter plates may be 24-well plates, 48-well plates or 96-well plates, however, other microtiter plates may be equally well suited and are also comprised by the invention.

Although the methodology described below is believed to contain sufficient details to enable one skilled in the art to practice the present invention, other constructs not specifically exemplified, such as plasmids, can be constructed and purified using standard recombinant DNA techniques as described in, for example, Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.).

As stated above, either a promoter or a response element thereof may be used for a method of the invention. Whereas every CYP gene is controlled by a unique promoter, genes which respond to identical inducers contain a common response element within their promoters. Accordingly, the same response element is responsible for inducing expression of a family of genes upon exposure to a certain compound. When isolated and operably linked to a minimal promoter and a structural gene, the resulting construct functions like a CYP promoter. This is particularly useful in dissecting a native CYP promoter that responds to multiple compounds into its component parts. The sequence of the promoter may not

always be precisely known, but often the general location of the promoter is known, for example, the promoter can be known to reside in a particular restriction fragment. Usually, the promoter or response element will include about 1 kb, but possibly more, of flanking genomic DNA at the 5' end of the transcribed region of the gene. The promoter region is useful for determining the pattern of expression, e.g. induction and inhibition of expression, and for providing promoters that mimic these native patterns of expression. Similarly, the response element suitable for use herein can be any response element to which induction or inhibition is desired. Examples of such response elements are as described herein and in the prior art. The response element herein may be part of the promoter sequence by conventional techniques such as by synthesis or excision of a known sequence by restriction enzyme and linked to the promoter sequence with or without the use of linkers.

In another embodiment, the genes operatively linked to the promoters are most preferably the native CYP genes. In this manner no genetic manipulation need be carried out on the genes prior to running an assay. In this preferred embodiment, the kits further comprise oligonucleotides or cDNAs which are complementary to at least a portion of either the coding or non-coding strand of the genes under control of the specific CYP promoters. The oligonucleotides are used to detect and quantify the mRNA transcripts of those genes or the cDNA complement thereof, either of which may be the detectable product in this embodiment.

The oligonucleotides employed in the diagnostic kits and methods of this invention are chosen based upon their ability to specifically hybridize under relatively high stringency conditions to either the transcription product of the gene operatively linked to the various CYP promoters or its complement (i.e., a single-stranded cDNA reverse transcribed from that mRNA). The choice of utilizing complementary or homologous oligonucleotides depends upon the method used for detecting the transcription products. These various methods are described later in the application. Because the DNA sequence of many CYP genes are known, hybridizable oligonucleotides are easily constructed. It should be noted that 100% homology or complementarity between the oligonucleotide and the CYP gene

mRNA is not required. This is because the oligonucleotide may be designed based upon the sequence of a CYP gene from a species different from the source of the cells utilized in the kits and methods of this invention. The oligonucleotides utilized in the kits and methods of this invention are preferably at least 95% homologous or complementary. Preferably, the oligonucleotides are between 20 and 500 base pairs long. Most preferably, the oligonucleotides are between 50 and 100 base pairs.

More preferably, the oligonucleotides are synthesized using an oligonucleotide synthesizer, optionally followed by polymerase chain reaction ("PCR"). In this procedure, an oligonucleotide having a sequence identical to a portion of either the template strand or the non-coding strand and within the coding region of a known, sequenced stress gene is synthesized. If PCR is to be used to increase the quantity of oligonucleotide, the oligonucleotide is synthesized with an additional 6 to 12 nucleotides at each end. Those extra nucleotides serve as targets for complementary primers in a PCR reaction. Preferably the extra nucleotides at each end are complementary to one another. This allows a single primer to prime off of both the original oligonucleotide and the PCR product thereof. Most preferably, the extra nucleotides at each end are complementary homohexamers, i.e., AAAAA at one end and TTTTTT at the other. During PCR, one or more labeled nucleotides are preferably included in the polymerase reaction. Suitable labels include fluorochromes, e.g. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-catoxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine JAMRA), radioactive labels, e.g. ^{32}P, ^{35}S, ^{3}H; etc. The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, etc. having a high affinity binding partner, e.g. avidin, specific antibodies, etc., where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The design of appropriate oligonucleotide probes for use in the kits and methods of this invention is relatively straightforward. Obviously, they should have high

sequence similarity or complementarity to the CYP gene mRNA to which they are designed to hybridize. The oligonucleotides in any particular kit should also have approximately the same melting temperature (Tm) so that a single warming apparatus (such as a water bath) may be utilized when carrying out hybridization and subsequent washing steps. Preferably the oligonucleotides are designed to have a Tm of greater than 70°C in 0.2X SSC. To determine which portions of the coding regions of the CYP gene to use in designing oligonucleotide probes, one may utilize a commercially available computer program, such as OLIGO (National Biosciences, Plymouth, MN).

The cells of the method or the kit of the invention may be cultured in a nutrient medium which supports and/or allows growth of said cells. Preferably, said medium comprises a charcoal stripped serum and thus is essentially free of steroids.
Further, said cell harbor a recombinant DNA molecule which comprises a heterologous promoter operatively linked to a gene encoding a first detectable product. Preferably, said heterologous promoter is constitutively active in said cells and is not influenced be the above specified compound suspected to be an inducer.
Examples for said heterologous promoter may be selected from the group of CMV-promoter, thymidine kinase-promoter, SV40-promoter, all of which are well known in the art, or promoters of "housekeeping genes" as defined infra.

Furthermore, the cells of the method or the kit of the invention may comprise at least one gene encoding a protein capable of regulating the expression of said drug metabolizing enzyme. As will be described infra in more detail, such genes may be the hPXR gene encoding an orphan nuclear receptor or genes encoding further nuclear receptors which may be required for induction of the CYP3A4 gene. By "at least one" it is meant that the cells may comprise up to a non-limited number of said different genes which may be required for CYP3A4 induction. Preferably, by said "at least one" gene a combination of hPXR and glucocorticoid receptor gene could be meant.

The cells of the method or the kit of the invention may harbor said at least one

gene either as recombinant DNA which has been transiently or stably introduced into said cells by methods which are well known in the art or as natural occurring gene in the genome. For the method of the invention it is important that the cells comprise suitable amounts of the protein encoded by said at least one gene. Therefore, it is preferred that said at least one gene is constitutively expressed or expression thereof is inducible by suitable means.

Moreover, the cells in the method or kit of this invention harbors a CYP3A4 promoter such as the promoter comprised by nucleotides –363 to +51 or –7830 to +51, or a CYP3A4 response element, such as the response elements comprised by nucleotides –7830 to –7209 or –7830 to +51, which is operatively linked to a heterologous gene encoding a second detectable product. It is preferable that the same heterologous gene be linked to the various CYP promoters or response elements. In this manner, only a single assay need be performed to detect induction of any of the CYP promoters and CYP response elements. It is also preferable that each cell contains only a single CYP promoter or response element/heterologous gene construct. Thus, the expression of the detectable product in any given cell can be specifically correlated to the induction of a single CYP promoter or response element. For embodiments of the invention that employ a heterologous gene operatively linked to a mammalian stress promoter or stress responsive element, the choice of gene is essentially limitless. The only parameters that are required are (1) that a DNA sequence encoding the assayable product has been characterized; and (2) that the product of the gene can be detected. Sufficient characterization includes knowledge of the entire coding sequence, availability of a genomic clone or knowledge of a sufficient number of restriction sites within the genomic DNA sequence to allow the gene to be manipulated so as to create an operative linkage to the stress promoter.

According to another embodiment of this invention, individual response elements of a promoter may be isolated and then operatively linked to a mammalian minimal promoter and to a gene which encodes a detectable product. Thus, expression of the detectable product in the presence of a compound is correlated with only one particular type of induction. A "minimal promoter" is a naturally occurring promoter

that has been weakened so that it is not 100% active. For example, a promoter in which all but a TATA box has been deleted, such as the minimal fos promoter, as described in Berkowitz et al. (1989) Mol. Cell. Biol. 5:4272-4281. Such constructs may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the gene.

In a particularly preferred embodiment of the method the present invention, said drug metabolizing enzyme is a cytochrome P450 (CYP) and even more preferred CYP3A4. Cytochromes P450 (P450s) form a gene superfamily that are involved in the metabolism of a variety of chemically diverse substances ranging from endogenous compounds to xenobiotics including drugs, carcinogens, and environmental pollutants. Although P450 regulation is still poorly understood, it is well known that some of the P450 proteins are induced several-fold by specific drugs. This may cause variability in enzymatic activity with different groups of patients producing unexpected pharmacological activity of some drugs as a result of drug-drug interactions (Wadhwa, Ther. Drug Monit. 9 (1987), 399-406). Knowledge of potential P450 inducibility by drug candidates, prior to drug development, would greatly enhance the ability to develop drugs that are free of P450-inducing properties. In the past, various animal species have been used as models to investigate P450 induction by drug candidates. The data obtained from such in vivo models has proven to be beneficial in assessing P450 induction. However, at the drug discovery stage this is difficult to carry out because of the large numbers of animals and the large amount of compound needed to conduct such experiments. In addition, there exist species differences in P450 induction (Kocarek, Drug Metab. Dispos. 23 (1994) 415-421), making the extrapolation from animals to humans unreliable. Therefore, having a simple, robust, and reproducible in vitro model to study P450 induction would greatly facilitate the ability to develop drugs devoid of these possible negative traits. Such a model would offer the advantage of requiring less drug (<1 mg) and reducing the number of animals used. In addition, confounding factors such as bioavailability, blood, and liver levels of the drug would be avoided. Previous attempts to set up an in vitro culture assay with hepatocytes failed for several reasons. As shown in the examples, the cell culture assay of the present invention provides a reliable test for putative CYP

inducers coming close to in vivo situation.

Genomic or cDNA clones encoding cytochrome P450 genes may be isolated using hybridization probes designed on the basis of the nucleotide or amino acid sequences for the desired gene. The probes can be constructed by chemical synthesis or by polymerase chain reactions using primers based upon sequence data to amplify DNA fragments from pools or libraries. The sequences of various cDNA isolates are described for cytochrome P4502C9 (Umbenhauer, Biochem. 26 (1987), 1094-1099, and Kimura, Nucl. Acids Res. 15 (1987) 10053-10054); P4502E1 (Song, J. Biol. Chem. 261 (1986), 16689-16697 and Umeno, Biochem. 27 (1988) 9006-9013); and P4503A4 (Beaune, Proc. Natl. Acad. Sci. U.S.A. 83 (1986), 8064-8068 and Gonzalez, DNA 7 (1988) 79-86). Cytochrome P450 1A2 is described by Jaiswal, Nucl. Acids Res. 14 (1986), 6773-6774; 2A3 by Yamano, Biochem. 29 (1990), 1322-1329; and 2D6 by Gonzalez, Genomics 2 (1988), 174-179. Genomic and cDNA sequences of the CYP3A4 gene are described, for example in Bork, J. Biol. Chem. 264 (1989), 910-919; Hashimoto, Eur. J. Biochem. 218 (1993), 585-595; Beaune, Proc. Natl. Acad. Sci. USA 83 (1986), 8064-8068; Molowa, Proc. Natl. Acad. Sci. USA 83 (1986), 5311-5315; Accession numbers: M14096, J04449, X12387, M18907. Genbank accession no. D11131 provides a partial sequence of the human cytochrome P-450IIIA4 gene. Genbank accession no. M18907 (cDNA) provides the cDNA sequence of a human CYP3A4 allele. A reference sequence for CYP3A7 is described in Komori, J. Biochem. 105 (1989), 161-163; Accession number: gi4503232 and a genomic sequence is described in Itho, Biochem Biophys Acta 1130 (1992), 133-138. For the hPXR gene a reference sequence is described in Bertilsson, Proc. Natl. Acad. Sci. USA. 95 (1998), 12208-12213; Lehmann, J. Clin. Invest. 102 (1998), 1016-1023; Accession numbers: AF061056, AF084645, AF084644.

In the following, the CYP3A gene family, which is the preferred subject in the method and kits of the present invention, is discussed in more detail. At least three functional CYP3A proteins exist in humans. The CYP3A4 monooxygenase is the predominant cytochrome P450 in human liver and small bowel. The protein displays a broad substrate specificity and it metabolizes more than 60% of all drugs that are currently in use, including contraceptive steroids, antidepressants,

benzodiazepines, immunosuppressive agents, imidazole antimicotics, and macrolide antibiotics (Cholerton, Trends Pharmacol. Sci. 13 (1992), 434-439, Ketter, J. Clin. Psychopharmacol. 15 (1995), 387-398). In addition, CYP3A4 plays a major role in the protection from environmental toxins. For example, the protein metabolizes aflatoxin B1, which has been implicated in the etiology of liver cancer, which is a major cause of premature death in many areas of Africa and Asia. Aflatoxin B1 is a mycotoxin produced by species of Aspergillus, and human exposure results principally from the ingestion of stored foodstuffs contaminated with the mold. Carcinogenicity is associated with its conversion to 8,9-oxide by the hepatic cytochrome P450-dependent monooxygenase system. Forrester et al. (Proc. Natl. Acad. Sci. U.S.A. 87 (1990), 8306-8310) found that the rates of metabolic activation of aflatoxin B1 were highly correlated with the level of proteins of the CYP3A gene family in the microsomes. Furthermore, Paolini et al. (Nature 398 (1999), 760-761) found significant increases in CYP3A in the lungs of rats treated with high doses of beta-carotene. Consequently, it was proposed that correspondingly high levels of CYP3A4 in humans would predispose an individual to cancer risk from the bioactivated tobacco-smoke procarcinogens, thus explaining the cocarcinogenic effect of beta-carotene in smokers. All this implies that the level of CYP3A4 activity could influence the efficacy of a variety of drug therapies as well as the individual predisposition to several major cancers caused by environmental carcinogens. A considerable variation in the CYP3A4 content and catalytic activity has been, indeed, described in the general population. For example, the metabolic clearance of the gene substrates exhibits a unimodal distribution with up to 20-fold interindividual variability. The activities of the CYP3A4 protein in liver biopsies vary up to 30-fold (Westlind, Biochem. Biophys. Res. Commun. 259 (1999), 201-205). Furthermore, many common drugs alter the expression levels of the gene and the extent of this phenomenon is individually variable. The inducers of CYP3A4 expression include commonly used drugs such as the glucocorticoid dexamethasone, the antibiotic rifampicin, and the antimycotic clotrimazole. More recently, a polymorphism (CYP3A4-V) has been described in the nifedipine-specific response element of the CYP3A4 promoter (Rebbeck, J. Natl. Cancer Inst. 90 (1998), 1225-1229). Its presence associates with a more advanced prostate tumor stage (Rebbeck, loc. cit.). The inducibility of CYP3A4

expression, combined with the diverse range of substrates, creates a potential for potentially harmful drug interactions involving this isozyme in patients undergoing therapies with multiple drugs.

CYP3A3 is a very closely related isoform to CYP3A4 (>more than 98% cDNA sequence similarity), but it is not known whether this reflects a separate gene product or an allelic variant. By contrast, CYP3A5 is a gene distinct from CYP3A4 and it is expressed polymorphically both in the adult and fetal liver and in the kidney and intestine. In adult Caucasians, the mRNA and the protein were detected in the liver of 10 to 30% of samples, while the protein was detected in the kidney and intestine of 70% of subjects (Jounaidi, Biochem. Biophys. Res. Commun. 221 (1996), 466-470 and references therein). CYP3A7 is the third functional CYP3A isoform. CYP3A7 was originally isolated from a fetal liver but it was subsequently found in 54% of adult livers (Schuetz, Pharmacogenetics 4 (1994), 11-20).

Tests to estimate the inducibility and the activity of CYP3A isozymes would be of obvious relevance for the optimization of therapies with drugs which are their substrates, and for the prevention of the associated side effects. Direct estimates of the activities of CYP3A isozymes in liver biopsies are possible but impracticable for both ethical and cost reasons. The indirect *in vivo* tests of CYP3A4 activity such as the erythromycin breath test or the 6-β-hydroxycortisol test pose ethical problems such as the invasive administration of undesirable probe substances and they are obviously unsuited for routine testing. In addition, the lack of correlation between these tests questions their informative value regarding the CYP3A4 activity (Hunt, Clin. Pharmacol. Ther. 51 (1992), 18-23). The methods and kits of the present invention are aiming at solving these problems.

As described in WO 97/08342 the use of cytochrome P450 coding sequence advantageously provides a method for measuring the activity of a promoter sequence in intact mammalian cells that contain a cytochrome P450 catalysis system using real time light (fluorescence) measurements. Thus, the use the entire CYP genomic sequences as the reporter gene or to use CYP coding sequences in combination with promoters or corresponding response elements of other CYP genes eliminates many of the labor-intensive aspects (e.g., cell lysis and

separation of cellular components) that generally are required to practice other methods for measuring the activity of a promoter sequence in a mammalian cell.

The term "qualifying" as used herein refers to determining the amount of the detectable products. It is very well known that ways of detection applied therefor are dependent on the nature of the products which are to be detected. For example, a detectable product may comprise a fluorescent or bioluminescent label which allows detection or quantification by a suitable device therefor. Alternatively, said detectable product may be an enzyme which generates a detectable read out which can be quantified by a suitable device. Usually, quantification may be assisted by automation.

The information on the induction profile of a drug gathered in accordance with a method of the present invention, preferably in combination with a patients genotype information can be used to predict the response of the individual to a particular CYP substrate or modulator. Where a modulator, e.g. a macrolide drug, inhibits CYP expression, then drugs that are a CYP substrate will be metabolized more slowly if the modulator is co-administered. Where a modulator induces CYP expression, a co-administered substrate will typically be metabolized more rapidly. Similarly, changes in CYP activity will affect the metabolism of an administered drug. The pharmacokinetic effect of the interaction will depend on the metabolite that is produced, *e.g.* a prodrug is metabolized to an active form, a drug is metabolized to an inactive form, an environmental compound is metabolized to a toxin, etc. Consideration is given to the route of administration, drug-drug interactions, drug dosage, etc.; see infra.

In a preferred embodiment of the method of the invention said method comprises the further steps of

(f) culturing cells of the cell line LS-174T (ATCC No. CI-188) or cells derived therefrom in a nutrient medium essentially free of steroids and capable of supporting the growth of said cells, wherein said cells comprise at least one gene encoding a protein capable of regulating the experession of said drug metabolizing enzyme and a first recombinant DNA molecule which

comprises a heterologous promoter operatively linked to a gene encoding a first detectable product and a second recombinant DNA molecule which comprises nucleotides –7830 to +51 or at least one repeat of nucleotides –7830 to –7209 and nucleotides –363 to +51 of the CYP3A4 gene (Accession number: AF185589) operatively linked to a gene encoding a second detectable product;

(g) incubating said cells with a second amount which is increased compared to the first amount of step (b) of a compound or a sample comprising a plurality of compunds suspected to be an inducer under conditions which permit expression of said first and said second detectable product;

(h) quantifying said first and said second detectable product;

(i) determining the ratio of the amount of said second and said first detectable product; and

(j) comparing the ratio determined in step (i) with the ratio obtained in step (i) of a corresponding assay with a control substance, wherein an increased ratio is indicative for a inducer;

(k) determining the ratio of the ratios characterized in step (e);

(l) determinig the ratio of the ratios characterized in step (j); and

(m) comparing the ratio determined in step (k) with the ratio determined in step (l), wherein an increase of the ratio determined in step (l) compared to the ratio determined in step (k) is indicative for an inducer.

In one embodiment of the present invention, said cell derived from LS-174T (ATCC No. CL-188) is obtained by introducing at least one gene encoding a protein capable of regulating the expression of a drug metabolizing enzyme into said cell, said gene being under the control of a promoter functional in the cell, whereby said gene is expressed. In a preferred embodiment of any one of the above described methods said gene regulating the expression of the drug metabolizing enzyme or drug transporter gene is an orphan nuclear receptor gene. Such orphan nuclear receptors bind for example to a cytochrome P-450 monooxygenase (CYP) promoter and usually are activated by compounds that induce CYP gene expression for example that of CYP3A4 which catalyzes the metabolism of >60% of all drugs that are in use including steroids, immunosuppressive agents,

imidazole antimycotics, and macrolide antibiotics (Maurel, P. in Cytochromes P450: metabolic and toxicoligcal aspects (ed. Ioannides, C.) 241-270 (CRC Press, Inc., Boca Raton, FL, 1996).

Expression of the CYP3A4 gene is markedly induced both *in vivo* and in primary hepatocytes in response to treatment with a variety of compounds. Many of the most efficacious inducers of CYP3A4 expression are commonly used drugs such as the glucocorticoid dexamethasone, the antibiotic rifampicin, the antimycotic clotrimatzole, and the hypocholesterolemic agent lovastatin (Maurel, P. in Cytochromes P450: metabolic and toxicological aspects (ed. Ionnaides, C.) 241-270 (CRC Press, Inc., Boca Raton, FL, 1996), Guzelian, P.S. in Microsomes and Drug Oxidations (eds. Miners, J.O., Birkett, D.J., Drew, R. & McManus, M.) 148-155 (Taylor and Francis, London, 1988). The inducibility of CYP3A4 expression levels coupled with the broad substrate specificity of the CYP3A4 protein represent the basis for many drug interactions in patients undergoing combination drug therapy. While attempts have been made to develop *in vivo* and *in vitro* assays with which to profile the effects of compounds on CYP3A expression levels, these efforts have been hampered by species-specific effects that have limited the utility of using animals and their tissues for testing purposes. Thus, analysis of the effects of new compounds on CYP3A4 gene expression has been largely restricted to laborious assays involving human liver tissue. Recently, efforts have been directed at understanding the molecular basis for the induction of CYP3A4 gene expression. The CYP3A4 promoter has been cloned and a 20 bp region residing approximately 150 bp upstream of the transcription initiation site shown to confer responsiveness to dexamethasone and rifampicin (Hashimoto, Eur. J. Biochem. 218 (1993), 585-595; Barwick, Molec. Pharmacol. 50 (1996), 10-16). This region contains two copies of the AG(G/T)TCA motif recognized by members of the nuclear receptor superfamily, suggesting that a nuclear receptor might be responsible for mediating at least some of the effects of the chemical inducers of CYP3A4 expression. A major breakthrough in the understanding of the CYP3A expression and inducibility took place in 1998 when three research groups independently suggested that the expression of CYP3A4 might be regulated by a member of the orphan nuclear receptor family termed PXR (pregnane X receptor),

or PAR (Lehmann, J. Clin. Invest. 102 (1998), 1016-1023; Bertilsson, Proc. Natl. Acad. Sci. U.S.A. 95 (1988), 12208-12213; Kliewer, Cell 92 (1998), 73-82). Furthermore, another research Group (Goodwin, Mol. Pharmaco (56/1999), 1329-1339) could show that PXR is indeed a regulator of CYP3A4. Upon treatment with inducers of CYP3A4, PXR binds to the rifampicin/dexamethasone response element in the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor (RXR). Northern blot analysis detected most abundant expression of hPXR in liver, colon, and small intestine, i.e. in the major organs expressing CYP3A4. The available evidence suggests that human PXR serves as a key transcriptional regulator of the CYP3A4 gene. A recent report describes the induction of CYP3A7 mediated by PXR suggesting that all members of the family may be regulated by this common transcriptional activator (Pascussi, Biochem. Biophys. Res. Commun. 260 (1999), 377-381). The use of the human pregnane X receptor (hPXR) makes possible assays that can be used to establish whether drugs will interact *in vivo*. For example, it might be advantageous to employ CYP3A4 and hPXR since a compound that affects the functional activity of PXR in a cell can be used to associate PXR activity with a mammalian disease state. For example, a compound that activates PXR induces CYP3A4. Thus, diseases in which CYP3A4 activity is important are associated with hPXR activity. Thus, in a preferred embodiment of the methods and kits of the invention said orphan nuclear receptor is the pregnane X receptor (PXR). Nucleotide sequences encoding hPXR, vectors, assays etc. that can be used and adapted in accordance with a method or kit of the present invention are described in for example WO 99/48915. Also comprised by the present invention are variants of hPXR, such as those comprising amino acid deletions, additions or substitutions.

The recombinant DNA molecule may be ligated into a shuttle vector through techniques known in the art. The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc. The recombinant DNA molecules can be introduced into the cells by conventional techniques including lipofectamine,

DEAE-dextran, electroporation, and calcium phosphate, and as described in the examples. Incorporation of the recombinant DNA molecule into a plasmid vector is optional and serves only to facilitate the construction of the recombinant DNA molecule, its transfection and selection. The cells could be transfected with naked recombinant DNA and laboriously screened for colonies containing recombinant DNA that had integrated into the host DNA and was functional. Vectors useful for introduction of the gene include plasmids and viral vectors, *e.g.* retroviral-based vectors, adenovirus vectors, etc. that are maintained transiently or stably in mammalian cells. A wide variety of vectors can be employed for transfection and/or integration of the gene into the genome of the cells. Alternatively, micro-injection may be employed, fusion, or the like for introduction of genes into a suitable host cell. The viral vector is preferably replication defective so that stable cell lines expressing for example CYP3A4 genes are obtained. For example, the cells may be infected with a molony-LTR driven promoter or a vaccinia virus or lipofected with an adenovirus promoter, HIV-promoter or CMV-promoter construct. The transfected DNA plasmid can contain a selectable marker gene or be co-transfected with a plasmid containing a selectable marker, and in some cases, the retroviral vector contains a selectable marker gene. Where one or more selectable marker is transferred into the cells along with the CYP3A4 gene, the cell populations containing the CYP3A4 gene can be identified and enriched by selecting for the marker or markers. Markers typically are antibiotic resistant to such antibiotics as tetracycline, hygromycin, neomycin, and the like.

In embodiments which employ a gene encoding a detectable product, the assayable product is preferably a polypeptide that is capable of providing a detectable signal either on its own upon transcription or translation or by reaction with another one or more reagents. Reporter genes suitable for use herein are conventional in the art, selection of which is within the capability of one skilled in the art. Examples of such reporter genes include that encoding the enzyme chloramphenicol acetyltransferase ("CAT"), the luc gene from the firefly that encodes luciferase, the Renilla luciferase gene, the bacterial lacZ gene from Escherichia coli that encodes beta-galactosidase, alkaline phosphatase ("AP"), human growth hormone ("hGH"), the bacterial beta-glucuronidase ("GUS"), and

green fluorescent protein ("GFP"), as described in Ausubel et al., Current Protocols in Molecular Biology (1994), (Greene Publishing Associates and John Wiley & Sons, New York, N.Y.). Most preferably, the *luc* gene is employed. If a genomic fragment containing a CYP3A4 promoter and its gene have been isolated or cloned into a vector, the promoter is removed by appropriate restriction enzyme digests. The promoter fragment is then isolated and operably linked to a gene encoding an assayable product in a plasmid. The vector should also contain a marker, such as Neo, for identifying stable transfectants. Screening for a functional fusion is achieved by exposing transfectants to an agent which is known to induce the specific CYP3A4 promoter and assaying for the detectable gene product. If the nucleotide sequence of the CYP3A4 promoter and its gene is known, polymerase chain reaction technology may be employed to produce assayable protein fusions. Specifically, one synthesizes primers which are complementary to the 5' and 3' ends of the CYP3A4 promoter portion of the gene, hybridizes those primers to denatured, total mammalian DNA under appropriate conditions and performs PCR. In this manner, clonable quantities of any sequenced CYP3A4 promoter may be obtained. Once the CYP3A4 promoter DNA has been obtained, it is operatively linked to a DNA encoding an assayable protein in an appropriate vector, as described above. Such methods are well-known in the art. Constructing operable fusions of CYP3A4 promoter response elements to a gene encoding a detectable product is also carried out by standard recombinant DNA techniques. Because response elements are small, DNA encoding them may be produced using an oligonucleotide synthesizer. Oligonucleotides corresponding to both strands of the response element are synthesized, annealed together and cloned into a plasmid containing a reporter gene under control of a minimal promoter. Alternatively, the double stranded oligonucleotides can be allowed to multimerize via self ligation prior to insertion into a vector. The multiple copies of the response element allow for higher expression of the detectable product upon induction. When utilizing CYP3A4 promoter-assayable product fusions, it is preferable that each cell employed in the kits and methods of this invention harbors only one such fusion. In this manner, if a compound induces expression of the assayable gene product in any particular cell, the specific type of CYP3A4 expression caused by the compound can unambiguously be identified.

In a preferred embodiment of the method of the invention the said first, the said second recombinant DNA molecule or gene is introduced into the cell by means of a retroviral vector.

In a furthermore preferred embodiment said first and said second detectable products are different.

In one embodiment of the above described methods, the test compound is a suspected agonist/activator of the activity of a CYP3A4 gene. In addition to the up-regulation of CY3A4P with inducing agents, it is also possible to modulate its activity with the use of compounds which can act as antagonists, i.e. down-regulators of transcription or enzyme activity. This allows the extinction of promoter activity at precise time points after activation by the administration of an antagonist. An antagonist would also prevent any opportunistic expression of the promoter due to uncontrolled environmental factors. Accordingly, in another embodiment of the method of the present invention, the substance to be tested is a suspected antagonist/inhibitor of the activity of a CYP3A4 gene. In the embodiments relating to methods for screening and identifying inhibitors of promoters or inhibitors of transcriptional activation of CYP3A4 genes the principle assay means described in US-A-5,747,338 may be employed. This patent describes constructs that provide for increased expression of reporter gene signal in the presence of an appropriate inhibitor and also describes methods for making and producing such constructs and vectors containing the same.

It is known that some compounds are not toxic to mammals in their native form, but become toxic after being processed by the liver. Therefore, according to another embodiment of this invention, the compound to be tested in the methods and kits of this invention is pre-treated with an S9 liver extract. Methods for preparing an S9 liver extract ("S9") are described by S. Vennitt et al., In Mutaqenicitv Testing - A Practical Encroach, S. Vennitt et al., eds., IRL Press, Oxford, England, pp. 52-57 (1984), the disclosure of which is herein incorporated by reference. S9 is generally a crude homogenate of rat liver with insoluble particles removed by low speed

centrifugation, but may also be prepared from human or other mammalian liver. S9 is incubated with the test compound in a potassium buffer containing NAD(P)H to mimic stage I and stage II biotransformation of compounds normally performed by the mammalian liver prior to performing the toxicity assay. If, however, primary mammalian liver cells are utilized in the kits and methods of this invention, S9 pre-treatment is unnecessary. The cells will be capable of performing stage I and II biotransformation of compounds under assay growth conditions. Alternatively, the cells utilized in the kits and methods of this invention are co-cultured with cells capable of performing stage I and II biotransformation, preferably, a primary liver cell line. The biotransformation of the compound being assayed is, in this instance, performed by those other cells, rather than enzymatic fractions derived from liver cells.

Prior to carrying out an assay on a compound of unknown potential inducing or antagonizing activity using the methods and kits of this invention, standard curves may be generated utilizing at least one and preferably at least three compounds that are known to induce the CYP3A4 promoter or response element that will be used to screen the unknown compound. Each known chemical should more preferably be tested against more than one, preferably three of the promoters, not just the promoter that it is known to induce. And each chemical should be assayed over a sufficiently wide range of concentrations to provide a useful standard curve, preferably 1 picomolar to 1 millimolar as well as at several time points. Once the standard curves have been generated, a computer data base containing those curves is generated. This database is then used to compare CYP3A4 promoter-induction profiles of the compounds to be tested with those of the known toxins used to generate the standard curve. Thus, the results for any untested compound are expressed in terms of relative toxicity compared to known inducers of CYP3A4 promoters. Each of the characterization and determination methods of this invention comprise the first step of culturing the cells both prior to and following exposure to a potential inducing compound.

Culture conditions will vary depending upon the compound type utilized. Growth of the cells is performed under standard tissue culture conditions in phenol red-free

Dulbecco's modified Eagle medium (DMEM) buffered with 25 mM HEPES and supplemented with 10% fetal calf serum that was pretreated with dextran-coated charcoal to remove any endogenous steroids as described (Walker and Enrietto, 1995), 100 IU/ml penicillin, 100 µg/ml streptomycin, 1% MEM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate. Cells are grown at 37°C with 5% CO_2 in a humidified incubator. The cells are routinely grown until they reach a density of about 5 x 10^5 cells/ml. Following this initial growth, the cells are subcultured and exposed to the compound to be tested in phenol red-free DMEM and supplemented with fetal calf serum that was pretreated with dextran-coated charcoal to remove any endogenous steroids as described (Walker and Enrietto, 1995).

A typical assay employs approximately 5 x 10^5 cells/ml. For initial tests on a compound, a series of 10-fold dilutions of the compound should be used. Another series of dilutions of the compound which have been pre-incubated with S9 fraction should also be prepared and added to a second portion of each culture. A third portion of each culture, which serves as a control, is not exposed to the compound, but otherwise treated in the same manner as described below. All of the cultures are then allowed to incubate at normal growth temperature for a period of time ranging from 5 minutes to 48 hours. More preferably, exposure to the test compound is for about 2 to 32 hours. Following exposure to the test compound, the level of assayable product or CYP gene mRNAs are measured. If the embodiment measuring assayable product is employed, quantification may be carried out in a number of ways that are well known in the art. For example, a colorimetric substrate may be utilized if the expression product is an enzyme. Appropriate colorimetric substrates for specific enzymes are well known in the art. Alternatively, an assay which employs specific antibodies, such as an RIA or ELISA, can be used to detect the expression product.

Depending upon the nature of the assay used, the buffer conditions of the lysed culture or supernatant may need to be adjusted. Accordingly, suitable buffer may be added to the lysed culture or supernatant so that optimal conditions for the particular assay are obtained. For example, if the assayable product is to be

detected by an RIA or ELISA assay, the buffer conditions must be adjusted to a neutral pH to allow for maximal antibody-antigen complex formation and to minimize non-specific antibody binding. Such conditions are well known in the art and are exemplified by a final buffer condition of 50 mM phosphate buffer, 150 mM NaCl, pH 7.0. If the assayable product is an enzyme and detection is to be achieved by a colorimetric substrate assay, buffer conditions must be optimized for maximal enzymatic activity and minimal non-catalytic cleavage of the substrate. These conditions are conventional and vary depending on the enzyme to be assayed.

In the most preferred embodiment of this aspect of the invention, the detectable product is luciferase (luc). Assays for this enzyme are well-known in the art and are described in J. Sambrook et al., "Molecular Cloning - A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, pp. 16.60-16.65 (1989), the disclosure of which is herein incorporated by reference. That reference also describes assays for beta-galactosidase, another assayable product useful in the methods and kits of this invention (pp. 16.66-16.67).

In embodiments that utilize transcription level to determine CYP gene induction, the level of mRNA transcribed from genes operatively linked to the CYP promoters utilized in the kits and methods of this invention must be measured ("CYP gene mRNA"). This requires that total RNA or mRNA be isolated from exposed cells. This may be achieved by any of the numerous and well-known methodologies. Commercially available mRNA or total RNA isolation kits may also be utilized, such as is available from Stratagene (La Jolla, CA). Preferably the cells are lysed with guanidinium isothiocyanate (GTC). The lysate is then acidified with sodium acetate buffer (pH 5.2) and the contaminants extracted with phenol. The RNA is then twice precipitated with ethanol, dried and redissolved in water. Once the RNA has been isolated, the level of CYP gene mRNA can be measured in a number of ways, either directly or indirectly. In the direct method, oligonucleotides that are complementary to CYP gene mRNA are used. In this method, the mRNA isolated from the cells is applied to nitrocellulose paper or nylon membrane filter in a slot blot apparatus. After diluting the RNA in the apparatus with appropriate salt

solution (preferably two volume of 20X SSC) and washing the slots, the nitrocellulose paper or filter is either baked at 80°C for 2 hours in a vacuum oven or UV crosslinked to fix the RNA. The RNA fixed to he nitrocellulose is then hybridized to labeled oligonucleotide probes which are complementary to CYP gene mRNAs under appropriate buffer and temperature conditions. An indirect method utilizes oligonucleotides that are homologous to CYP gene mRNAs for detection. This method measures transcription by using the CYP gene mRNAs as templates for making labeled single stranded cDNA using reverse transcription. These cDNAs are then detected and quantitated by hybridizing to complementary oligonucleotides (or denatured double-stranded cDNAs) that are bound to a solid support. Preferably, the solid support is a negatively charged membrane and the oligonucleotides are modified by the addition of a positively charged amidite or amino group on the 3' end prior to binding to the membrane. This 3' modification allows the oligonucleotide to bind to the membrane only via its 3' end, allowing for more efficient hybridization than other methods of binding DNA to a solid support.

In either method, a control representing a constitutively expressed "housekeeping gene", such, gapdh, α -globin, β-tubulin, β-actin or actin, which is not induced by the specific experimental sample, is also used. This provides a control for proper growth and functioning of the cells, as well as the background standard upon which to calculate the amount of specific induction. Following hybridization, the amount of hybridization is quantified. Quantification is achieved by a method that is consistent with the label on the oligonucleotide or cDNA. If a radioisotope is used as a label, the membrane is exposed in a phosphoimager for quantification as the preferred method. Exposure of the membrane to X-ray film followed by densitometry tracing or liquid scintillation counting would be still other preferred methods of quantification. If a fluorescent label is used, a fluorometer is used for quantification. In this manner the level of various stress gene inductions can be measured. If a biotinylated label is used, quantification is achieved by using streptavidin conjugated to an enzyme that can yield a measurable colorimetric product. In a preferred embodiment of the present invention, the above described methods comprise PCR, ligase chain reaction, restriction digestion, direct sequencing, nucleic acid amplification techniques, hybridization techniques or immunoassays

(Sambrook et al., loc. cit.). In one embodiment of the methods and kits of the present invention, an array of oligonucleotides is employed, where discrete positions on the array are complementary to one or more of the sequences of the detectable product, e.g. oligonucleotides of at least 12 nt. frequently 20 nt. or larger. Such an array may comprise a series of oligonucleotides, each of which can specifically hybridize to a different detectable product. For examples of arrays, see Hacia, Nature Genetics 14 (1996), 441-447; Lockhart, Nature Biotechnol. 14 (1996), 1675-1680; and De Risi, Nature Genetics 14 (1996), 457-460. The person skilled in the art, of course, knows that corresponding arrays can be produce which recognize proteins or other chemical compounds, for example substrates and/or products of a reaction that is catalyzed by the reporter gene or molecules that bind and label the detectable product. Accordingly, the present invention also relates to the use of array technology in accordance with a method of the present invention. Further types arrays are known to the person skilled in the art and are described for example in WO 97/49989. One embodiment of the arrays described in WO97/49989 is named XNA on Gold™ which is the registered trade mark of the affinity array biochip developed by INTERACTIVA Biotechnologie GmbH; see http://www.interactiva.de.

Induction or antagonizing potentials of compounds studied in the LS174T cell model can be defined as a percentage induction (or suppression) relative to a control compound such as rifampicin, which was included as a standard inducer in the experiments described in the examples. This enables comparison of induction or antagonizing potentials of compounds in the cell cultures, also with results obtained from different assays. Furthermore, variability may be minimized by expressing for example induction as a percentage of a standard inducer. This allows for rank ordering of compounds and provides a strategy for circumventing inter-individual variability. This in vitro assay and calculation approach correlated well with in vivo induction. In conclusion, this assay allows for numerous compounds to be evaluated at the drug discovery stage using minimal quantities of drug and markedly fewer animals.

It is known that while individual compounds may not be inducing or antagonizing,

combinations of non-active compounds may, in fact be modulators of CYP gene expression. Therefore, it should be understood that the kits and methods of this invention can also be utilized to determine the potential modulating activity of combinations of known and unknown compounds (e.g. drug interactions) in an identical manner to that described above.

According to another embodiment, the invention provides a method of identifying an inhibitor/antagonists to a compound determined to be an inducer by the methods of this invention. The cells used for the method of the invention may be pre-incubated with varying concentrations of the proposed antagonist prior to the addition of an inducing concentration of compound determined to be an inducer. If pre-incubation with the proposed antagonist decreases or obliterates the effect of the compound determined to be an inducer, such an antagonist will likely be effective. Thus, the present invention relates to a method of identifying and obtaining an inhibitor/antagonist to a new inducer of the activity of a drug metabolizing enzyme comprising the steps of:

(a) determining whether a compound suspected to be an inducer is an inducer using any one of the above described methods;

(b) repeating the method used in (a) with the additional step of treating the cells employed in said method prior simultaneously or after incubating the cells within the inducer identified in step (a) but prior, to quantifying a detectable product as specified in the above described methods, with a substance suspected to be an inhibitor/antagonist to said inducer identified in step (a); and

(c) determining whether the substance suspected to be an inhibitor/antagonist decreases or obliterates the effect of the inducer identified in step (a), wherein a decrease or obliteration is indicative for an inhibitor/antagonist.

Finally, this invention provides a method of improving active drug design. According to this embodiment, a new drug is first tested with any of the above-described kits and methods and its inducing activity is determined. The information provided by such methods and kits indicates the induction profile. The portion of the drug that is likely to cause the particular induction of CYP3A4 genes indicated

may then be appropriately modified or eliminated depending upon the role that portion plays in the drug's pharmaceutical activity. The resulting modified drug is then retested with the kits and methods of this invention to determine if its inducing capabilities have been sufficiently reduced or eliminated. Drugs improved and modified by this method are also within the scope of this invention. Preferably, the drugs obtained by the method of the invention are less toxic than the original drug or compound. Accordingly, the present invention relates to a method of decreasing the toxicity of a drug, comprising the steps of:

(a) determining the type of modulation caused by said drug using the methods according to the invention; and

(b) modifying said drug to alter or eliminate the portion thereof suspected of causing said determined modulation.

Said method may further comprise, after step (b), the additional step of:

(c) repeating the method used to determine the type of modulation caused by said drug according to step (a) using the modified drug according to step (b).

In one embodiment of the method of the invention said drug to be tested and modified are drugs such as Verapamil, Cyclosporin A or Erythromycin.

In accordance with the above, the present invention also relates to a modified drug produced by any one of the above described methods.

In a further embodiment the invention relates to a method for the production of a pharmaceutical composition comprising the steps of any one of the above described methods and synthesizing and/or formulating the compound identified as a new modulator or a derivative or homologue thereof in a pharmaceutically acceptable form. The therapeutically useful compounds identified according to the method of the invention may be formulated and administered to a patient as discussed above. For uses and therapeutic doses determined to be appropriate by one skilled in the art see infra. Furthermore, the present invention relates to a method for the preparation of a pharmaceutical composition comprising the steps of the above-described methods; and formulating a drug or pro-drug in the form suitable for therapeutic application. Drugs or pro-drugs after their *in vivo*

administration are metabolized in order to be eliminated either by excretion or by metabolism to one or more active or inactive metabolites (Meyer, J. Pharmacokinet. Biopharm. 24 (1996), 449-459). Thus, rather than using the actual compound or inhibitor identified and obtained in accordance with the methods of the present invention a corresponding formulation as a pro-drug can be used which is converted into its active in the patient. Precautionary measures that may be taken for the application of pro-drugs and drugs are described in the literature; see, for review, Ozama, J. Toxicol. Sci. 21 (1996), 323-329).

Moreover, the present invention relates to a composition, preferably pharmaceutical composition obtainable by the above described methods comprising the modulator, inhibitor or modified drug of the present invention, and optionally a pharmaceutically acceptable carrier. These pharmaceutical compositions comprising, e.g., the modulator or inhibitor or pharmaceutically acceptable salts thereof may conveniently be administered by any of the routes conventionally used for drug administration, for instance, orally, topically, parenterally or by inhalation. Acceptable salts comprise acetate, methylester, HCl, sulfate, chloride and the like. The compounds may be administered in conventional dosage forms prepared by combining the drugs with standard pharmaceutical carriers according to conventional procedures. These procedures may involve mixing, granulating and compressing or dissolving the ingredients as appropriate to the desired preparation. It will be appreciated that the form and character of the pharmaceutically acceptable character or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. The pharmaceutical carrier employed may be, for example, either a solid or liquid. Exemplary of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary of liquid carriers are phosphate buffered saline solution, syrup, oil such as peanut oil and olive oil, water, emulsions, various types of wetting agents, sterile solutions and the like. Similarly, the carrier or diluent may include time delay material well known to the art, such as glyceryl mono-stearate or glyceryl distearate

alone or with a wax. When the compounds identified, modified and synthesized in accordance with the methods of the present invention care used as pharmaceutical compositions, they can be administered either orally in the dosage form such as tablets, capsules, elixirs, microcapsules, or parenterally as injections in the form of solutions in water or in other pharmaceutically acceptable liquids, or suspensions. For example, the compounds can be formulated into the above dosage forms by mixing them with physiologically acceptable carriers, flavors, excipients, stabilizers, etc., in a generally accepted form. Additives that can be mixed in tablets include binders such as gelatin, swelling agents such as cornstarch, excipients such as crystalline cellulose, and lubricants such as magnesium stearate. In the case of capsules, liquid carriers can be contained in addition to the components described above. Sterile compositions for injection can also be formulated in the conventional manner. An aqueous solution for injection is exemplified by an isotonic solution containing glucose, which may also be combined with appropriate solubilizers such as polyethylene glycol. It may further contain buffers, stabilizers, preservatives, antioxidants, and soothing agents. The pharmaceutical preparations thus produced can be administered, for example, to humans and other mammals. Though the dose may vary depending on the symptoms and the like, the preparation is generally administered to an adult about 0.01 mg to about 100 mg, preferably about 0.1 mg to about 50 mg, more preferably - about 1.0 mg to about 25 mg per day in the case of oral administration. In the case of parenteral administration, for example, in the form of injection, an adult is generally intravenously given about 0.001 mg to about 50 mg, preferably about 0.01 mg to about 25 mg, more preferably about 0.1 mg to about 10 mg per day. The dosage regimen will be determined by the attending physician and other clinical factors; preferably in accordance with any one of the above described methods. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Progress can be monitored by periodic assessment.

The present invention also relates to the use of cells selected from the cell lines consisting of LS-174T (ATCC No. CL-188) and cells derived therefrom for the determination of whether a substance is modulator of the expression activity of a drug metabolizing enzyme or drug transporter gene or of a gene capable of regulating the expression of such a gene. Furthermore, the present invention provides a test kit useful for carrying out any one of the above described methods, which kit comprises:

(a) culturing cells of the cell line LS-174T (ATCC No. Cl-188) or cells derived therefrom, in a nutrient medium essentially free of steroids and capable of supporting the growth of said cells, wherein said cells comprise at least one gene encoding a protein capable of regulating the expression of said drug metabolizing enzyme and a first recombinant DNA molecule which comprises a heterologous promoter operatively linked to a gene encoding a first detectable product and a second recombinant DNA molecule which comprises nucleotides –7830 to +51 or at least one repeat of nucleotides –7830 to –7209 and nucleotides –363 to +51 of the CYP3A4 gene (Accession number: AF185589) operatively linked to a gene encoding a second detectable product; optionally in combination with

(b) a control substance and/or means and/or instructions for carrying out any one of the methods of the present invention.

The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic cells. The kit of the invention may advantageously be used for carrying out a method of the invention and could be, inter alia, employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit may be used for methods for detecting inducers of, e.g., CYP3A4 or hPXR gene in accordance with any one of the above-described methods of the invention, employing, for example, immunoassay techniques such as radioimmunoassay or enzymeimmunoassay or preferably nucleic acid hybridization and/or amplification techniques such as those

described herein before and in the examples. Preferably, the invention provides CYP-specific diagnostic kits and methods. For example, the invention provides CYP3A kits and methods. The choice of promoters to use in these CYP3A-specific kits may be made from any of the appropriate promoters of CYP3A genes described above. Preferably these kits employ at least 3, and more preferably at least 5, promoters which respond to different subsets of inducers within the larger group. Most preferably, these specific kits and methods employ at least 10 promoters of CYP genes. These kits and methods allow a more precise and specific analysis of the induction of subset of CYP genes caused by a compound.

These and other embodiments are disclosed or are obvious from and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on Internet, e.g. under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The invention will now be described by reference to the following examples which are merely illustrative. In particular, other promoters or response elements and other reporter gene can be substituted for the ones described herein. Further objects, features, and advantages of the present invention will become apparent from the following examples. It should be understood, however, that the examples are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. Certain of the basic molecular biology techniques described below are not set forth in detail. Such techniques are well

known in the art and are described in the disclosure of Molecular Cloning - A Laboratory Manual Second Edition, J. Sambrook et al., eds., Cold Spring Harbor Laboratory Press, New York (1989) or Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1994), the disclosure of which is herein incorporated by reference. Efforts have been made to ensure accuracy with respect to the numbers used (e.g. amounts, temperature, concentrations, etc.) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees centigrade; and pressure is at or near atmospheric.

Examples

Materials and reagents

Chemicals were obtained from Sigma (Deisenhofen, Germany) if not noted otherwise. Rifampicin was supplied by Merck (Darmstadt, Germany). Galacton™ and Emerald™ enhancer were supplied by Tropix (Bedford, Massachusetts, USA). Oligonucleotides were synthesized by MWG (Ebersberg, Germany). Radioactive isotope (α-^{32}P)dCTP was obtained from ICN (Eschwege, Germany). All cell culture media and supplements were derived from Life Technologies (Eggenstein, Germany).

Cell lines

The human colon adenocarcinoma cell lines LS180 and LS174T (Tom et al., 1976) were obtained from American Type Culture Collection (Manassas, Virginia, USA). Both lines were grown in Dulbecco´s modified Eagle medium (DMEM) buffered with 25 mM HEPES and supplemented with 10% charcoal stripped fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 % MEM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate. Cells were grown at 37 °C with 5 % CO_2 in a humidified incubator. COS-1 cells, HepG2 cells and Caco-2 TC7 cells were derived from the stock of the Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology. BN cells are described in Pang et al., 1996. COS-1 cells were cultured in DMEM buffered with 25 mM HEPES and supplemented with 10 % fetal

calf serum (charcoal stripped), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine. HepG2 cells were cultured in MEM supplemented with 10 % fetal calf serum (charcoal stripped), 2 mM L-glutamine and antibiotics as described above. Caco-2 TC7 and BN cells were grown in the same culture medium as LS-174T cells.

Eukaryotic expression plasmids

The open reading frame of human PXR was amplified by PCR out of cDNA of LS180 cells using the oligonucleotide primers 5´-TCGAATTCCACCATGGAGGTGAGACCCAAAGAAAGC-3' (SEQ ID NO: 1) and 5´-CGTCTAGATCAGCTACCTGTGATGCCGAACA-3' (SEQ ID NO: 2), which introduce EcoRI and XbaI sites respectively. The PCR fragment was digested with EcoRI and XbaI, cloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, Netherlands) to generate pcDhuPXR and sequenced.

Reporter genes

The CYP3A4 promoter region encompassing the sequence between –1105 and +51 with respect to the transcriptional start site (Hashimoto et al., 1993) was amplified by PCR out of human genomic DNA using the primers 5´-ATGGTACCTGCAGTGACCACTGCCCCAT-3' (SEQ ID NO: 3) and 5´-ACAAGCTTGCTCTTTGCTGGGCTATGTGCA-3' (SEQ ID NO: 4), which introduce KpnI and HindIII sites respectively. The PCR fragment was digested with KpnI and HindIII, cloned into the reporter gene vector pGL3-Basic (Promega, Mannheim, Germany) to generate pGL3-CYP3A4(-1105) and sequenced. Unidirectional deletion mutants were constructed using the double-stranded Nested Deletion kit (Amersham Pharmacia, Freiburg, Germany). The precise starting point of the deletion mutants was determined by sequencing. The internal deletion of sequence –250 to –210 and the point mutants of the ER6 motif were generated by sequential PCR steps as described (Higuchi, 1990) using appropriate primers and sequenced. Mutation of the ER6-motif was done according to published data (Lehmann et al., 1998).

The distal enhancer module located between –7830 and –7209 (Goodwin, Mol Pharmacol 56 (1999), 1329-39) was amplified by PCR out of a BAC clone containing human CYP3A4 gene (Genome Systems clone GS23577) using the primers 5' – GCG GTA CCG AGA TGG TTC ATT CCT TTC AT –3' (SEQ ID NO: 8) and 5' – CGA GAT CTC GTC AAC AGG TTA AAG GAG AA –3' (SEQ ID NO: 9) which introduce KpnI and Bg1II sites, respectively, into the amplified fragment. The fragment was then cloned into the KpnI and Bg1II sites of pGL3-CYP3A4(-1105) to generate the pGL3-CYP3A4(-7830Δ7208-364) plasmid which encompasses the nucleotides –7830 to –7209 and –363 to +51 of the CYP3A4 promoter.

Transient transfections, luciferase and β-galactosidase assays

Cells were seeded 24h prior to transfection to provide cell density of approximately 40% at the time of transfection. The plasmid DNA for transfection was prepared using Qiagen Plasmid Kits (Qiagen) according to manufacturer's instructions. Transfections were done 2-4 times in triplicate with at least two different plasmid DNA preparations.

For example, if 100 000 cells were seeded in a 24-well microtiter plate 0.1 μg reporter gene construct, 0.1 μg PXR expression plasmid and 0.1 μg of a mammalian ß-Galactosidase expression plasmid would be applied. Seven hours after transfection cells were treated with inducers, noninducers or DMSO. The majority of compounds were prepared as 1000x sock solutions in DMSO. Cells were harvested 48 hours after transfection, lysed with reporter lysis buffer (Promega, Mannheim, Germany) for 15 min and frozen for at least 30 min by –80°C. After thawing, lysates were used for reporter gene assays. Luciferase activities were determined on cell lysates using a commercially available assay system (Promega). β-Galactosidase assays were performed with chlorophenol red-β–D-galactosidase (CPRG, Roche, Mannheim, Germany) as the substrate. Luciferase activity was normalized with respect to transfection efficiencies using the corresponding β-galactosidase activity.

Treatment with inducers

Stock solutions (1000x) of the inducers were prepared in DMSO. Inducers and the DMSO solvent control were added to the cell culture medium to a final concentration of 0.1%. In induction experiments cells were cultured in phenol red-free DMEM and supplemented with fetal calf serum that was pretreated with dextran-coated charcoal to remove any endogenous steroids as described (Walker and Enrietto, 1995).

RNA preparation and Northern blotting

Preparation of polyadenylated RNA and Northern Blot analysis was done as described (Burk et al., 1993). Radioactive probes were synthesized using the DECAprime II Kit (Ambion, Austin, Texas, USA). To synthesize a probe specific for CYP3A4, the CYP3A4 cDNA fragment of pUVI-CYP3A4 (Kindly provided by F. J. Gonzalez) was used. The GAPDH-mouse DECAtemplate (Ambion) was used as a probe specific for gapdh. A PXR specific probe was synthesized using the PXR cDNA fragment of pcDhuPXR.

Protein analysis

COS-1 cells transfected as described above with pcDhuPXR for protein analysis were harvested and cell pellets boiled in protein sample buffer (62,5 mM Tris-Cl pH 6.8 / 10 % (v/v) glycerol / 2 % (w/v) SDS / 5 % (v/v) 2-mercaptoethanol / 0.01 % (w/v) bromophenol blue). Total cellular proteins were fractionated in a 10% SDS-polyacrylamide gel. Western blotting was performed as described by Klempnauer et al. (1986) and PXR protein detected with a polyclonal rabbit antiserum directed against the peptide ALIKRKKSERTGTQP (SEQ ID NO: 5), amino acids 123-137 of human PXR (Lehmann et al., 1998). PXR protein-antibody complexes were visualized using the ECL detection system (Amersham Pharmacia, Freiburg, Germany).

Example 1: Establishing a cell suitable for cytochrome P450 gene expression studies

Studying the molecular mechanisms of cytochrome P450 gene expression, for example intestinal CYP3A4 induction by rifampicin required at first to establish an appropriate cell culture model. Schuetz at al. (1996a) have shown that mRNA expression of endogenous CYP3A4 can be induced by rifampicin and reserpine in the colon carcinoma cell line LS180. But the specific culture conditions of LS180 makes this cell line unsuitable for promoter studies, which require transient transfection experiments to be done. Therefore different cell lines were searched for showing induction of CYP3A4 by rifampicin. After testing various collections of cells and cell lines LS174T was the only cell line that could be identified, which showed an induction. Northern blot analysis of mRNA of LS174T and LS180 cells treated for increasing time with rifampicin surprisingly showed that the induction of CYP3A4 in LS174T was even stronger and reached maximum levels earlier than in LS180 (Fig. 1A). The extent of induction was dependent on the concentration of rifampicin used. A range of concentrations between 0.1 μM and 10 μM rifampicin was tested. Starting with 0.5 μM rifampicin, where induction was detectable for the first time, CYP3A4 induction showed a concentration dependent increase (Fig. 1B). Because of the high degree of sequence homology between the human members of the cytochrome P450 subfamily 3A, it was confirmed by gene-specific PCR that it was really CYP3A4 that was induced. Induction of endogenous CYP3A4 in LS174T guaranteed that all the necessary transacting factors were provided by the cells. Therefore the colon carcinoma cell line LS174T was selected as a model system.

Example 2: Specific induction of exogenous cytochrome P450 promoter in LS174 cells

As the most likely mechanism of induction of CYP genes is a transcriptional one, we decided to analyze the induction by rifampicin with the help of CYP3A4

promoter reporter genes. The known regulatory region of CYP3A4, encompassing –1105 bp upstream of the transcriptional start site (Hashimoto et al., 1993) was cloned in front of a luciferase reporter gene. Transient transfection of this longest CYP3A4 promoter reporter gene in LS174T cells showed that it contained sequences mediating rifampicin induction (Fig. 2). But the amount of induction of the CYP3A4 promoter was considerably lower than induction of the endogenous CYP3A4 mRNA. Densitometric evaluation of the Northern blot shown in Figure 1A, revealed that the endogenous RNA in LS174T cells was induced approximately 40 fold after a two days treatment with rifampicin, against which the promoter construct was only induced 3 to 4 fold.

A reason for the observed stronger induction of the endogenous gene could be that not all relevant response elements are comprised by the promoter fragment. Goodwin et al. (Mol. Pharmacol 56 (1999), 1329-1339) have demonstrated existence of further upstream located nuclear response elements capable of binding PXR and involved in induction by rifampicin and other substances. Using these additional sequences it might be possible that induction by rifampicin is further stimulated also in LS174T cells.

Example 3: Promoter regions involved in cytochrome P450 gene regulation

To screen systematically for sequence elements mediating rifampicin induction, a series of unidirectional deletion mutants of the CYP3A4 promoter was constructed and tested in transient transfection experiments in LS174T cells. Deletion of sequences upstream of –270 did not alter the extent of rifampicin induction. A further deletion to –192 showed a slightly diminished induction. Deletion mutants beyond position –192 were no longer inducible by rifampicin (Fig. 2). So the only region mediating rifampicin induction in the known promoter of CYP3A4 is located between –192 and –136, with a possible minor contribution of sequences between –270 and –192. This latter region contained a set of closely spaced imperfect GRE half sites (TGTTCT) between –250 and -210. TGTTCT half sites have been shown to be present in a dexamethasone responsive enhancer of CYP3A5 and to be essential for induction (Schuetz et al., 1996b). So a mutant of the promoter was

constructed in which the region between -250 and –210 had been deleted. Transient transfection of the mutant showed that this region was not required for rifampicin induction (Fig. 3). The region that was identified to be essential for rifampicin induction in LS174T cells (-192 to –136) contained the previously described consensus region II which has been implicated in glucocorticoid and rifampicin induction in liver cells (Barwick et al., 1996). A palindromic consensus binding site for nuclear receptors ($AG^{G}/_{T}TCA$ repeat), an ER6 motif, is located in that sequence (Lehmann et al., 1998). A point mutation of the ER6 motif was constructed, according to published data, so that nuclear receptors were no longer able to bind to it (Lehmann et al., 1998). Transient transfection of this mutant showed that a functional ER6 motif is necessary for rifampicin induction of the CYP3A4 promoter (Fig. 3). So the only sequence element necessary for rifampicin induction that could be identified in the known 1105 bp of the CYP3A4 upstream regulatory region is a distinct binding site (ER6 motif) for nuclear receptors.

Example 4: Involvement of the Pregnane X Receptor (PXR) in the regulation of CYP expression

The question arose what the nuclear receptors are that mediate rifampicin induction through this element. The nuclear receptors PXR and CAR have been shown to be able to bind to the ER6 motif of CYP3A4 (Lehmann et al., 1998 / Sueyoshi et al., 1999). Furthermore the nuclear receptor COUP-TF I has been shown to bind to the corresponding element in rat CYP3A23 (Huss and Kasper, 1998), which differs only in one nucleotide from the ER6 motif of CYP3A4. So COUP-TF I would also be expected to bind to the ER6 motif of CYP3A4. While COUP-TF I is expressed in LS174T cells, CAR is not expressed at detectable levels. The possible involvement of COUP-TF I in regulation of CYP3A4 remains to be clarified. The recently identified nuclear receptor PXR has been shown to be activated by rifampicin (Lehmann et al., 1998), therefore it was first examined whether PXR could be involved in the rifampicin induction of the CYP3A4 promoter in LS174T cells. As is shown in Fig. 4A, PXR mRNA expression was abundant only in LS180 and LS174T cells, the only cell lines where rifampicin induction of

CYP3A4 could be observed. PXR expression was not detectable in the intestinal crypt cell-like line BN and only weakly in differentiated Caco-2 TC7 cells. The human hepatoma cell line HepG2 expressed PXR at remarkably lower levels. CYP3A4 could not be induced by rifampicin in these cell lines. So high expression of PXR correlates with inducibility of CYP3A4 by rifampicin. To further demonstrate an involvement of PXR in CYP3A4 induction by rifampicin, an eukaryotic expression plasmid for human PXR was constructed. The Western Blot shown in Figure 4B confirmed that the plasmid produced a protein of the expected molecular weight (50 kDa) which was recognized by an antibody directed against human PXR. Co-transfection of the human PXR expression plasmid together with a CYP3A4 promoter reporter gene into LS174T cells activated the CYP3A4 promoter already in the absence of rifampicin. Treatment with rifampicin then strongly induced the CYP3A4 promoter (Fig. 4C). This clearly demonstrated that exogenous PXR could function as a rifampicin dependent transcriptional activator of the CYP3A4 promoter in LS174T cells. It is therefore very likely that the endogenous transcriptional activator mediating rifampicin induction in LS174T cells is identical with PXR. It was not possible to demonstrate the existence of endogenous PXR protein in LS174T cells, possibly because of a modification of the PXR protein in LS174T cells in the region of the protein the antibody is recognizing. The region against which the antibody was directed contained consensus phosphorylation sites for cAMP dependent kinase and protein kinase C.

Example 5: Assay for screening inducers or inhibitors of CYP genes

Having demonstrated in Examples 1 to 4 that the CYP3A4 promoter reporter gene is inducible by rifampicin like the endogenous CYP3A4 gene, it was tested whether LS174T cells would be a suitable model system to screen for CYP3A4 inducers. First LS174T cells were treated with a panel of known CYP3A4 inducers or PXR activators and looked for induction of the endogenous CYP3A4 mRNA in Northern blots. Figure 5 shows that beside rifampicin, the known inducers RU486, clotrimazole, nifedipine and reserpine were inducing the endogenous CYP3A4 mRNA. Other known inducers like dexamethasone, PCN or carbamazepine were

not inducing. Also not inducing were PXR activators like pregnenolone, progesterone and cortisol. On the other hand the PXR activator corticosterone was also weakly inducing. Then LS174T cells were co-transfected with a CYP3A4 promoter reporter gene and PXR expression plasmids and treated the cells with the same compounds. With the exception of RU486, all compounds inducing the endogenous gene, also induced the CYP3A4 promoter reporter gene (Fig. 6). Out of all the compounds not inducing the endogenous CYP3A4 gene, only carbamazepine induced the CYP3A4 promoter reporter gene. So within the limitations of the cell line, activation of the promoter reporter gene reflects induction of CYP3A4, notwithstanding the non-inducibility observed for some known inducers like dexamethasone and PCN. Therefore the cell line LS174T provides a useful model system to screen for compounds inducing CYP3A4. The only exception was RU486, which induced the endogenous CYP3A4 gene, but failed to induce the promoter reporter gene. However, this problem may be overcome be generating promoter reporter gene plasmids comprising larger promoter regions. Preferably, said promoter reporter gene plasmids may contain the additional CYP3A4 sequences described by Goodwin (Mol. Pharmacol 56 (1999), 1329-1339). LS174T cells which are stably transfected with a promoter reporter gene construct comprising said larger promoter regions may be a suitable tool for an easy automatic assay. Moreover, said stably transfected cell line allows cotransfection of PXR as an enhancer for the assay.

Example 6: Improved assays for screening inducers of CYP genes

Combined assay for transfection efficiency

LS174T cells were transfected with the reporter gene construct pGL3-CYP3A4(-7830Δ7208-364), the expression plasmid pcDhuPXR and a mammalian ß-Galactosidase expression plasmid. Seven hours after transfection, cells were induced with different concentration of rifampicin.

The triplicates which are shown in the graph in Figure 7 resulted from the induction with 10 μM Rifampicin. They present the luciferase activity of cell extracts from

LS174T cells with and without normalizing with β-Galactosidase activity. The first three columns present the absolute luciferase activity (without compensation), the last three columns the relative luciferase activity which was normalized with respect to transfection efficiencies using the corresponding β-galactosidase activity. The absolute luciferase activity within the triplicate shows sizably discrepancy. After normalizing with β-galactosidase activity, the same values show very similar results. These results support the importance of the transfection efficiency and show the necessity of normalizing through combining luciferase and ß-Galactosidase activity. Evaluation without consideration the transfection efficiency may lead to wrong results including false negatives.

Combined assay of cytotoxic and/or stress effects

Treatment with some compounds may result in cell death due to cytotoxic or stress effects. In this case, the luciferase activity decreases because the number of living cells also declines. The cytotoxic or stress effects can be compensated by measuring of ß-Galactosidase activity which is indicative for the number of viable cells.

LS174T cells were transfected with the reporter gene construct pGL-CYP3A4(-7830Δ7208-364), the expression plasmid pcDhuPXR and a mammalian ß-Galactosidase expression plasmid. Seven hours after transfection, cells were induced with different concentration of RU 486.

Figure 8 presents the comparison of the CYP3A4 induction by RU 486 with or without the consideration of the ß galactosidase activity. This Figure demonstrates the necessity for consideration of cytotoxic or stress effects.

Combined assay of solvent effects

Most of the compounds which are used in induction assays are hydrophobic, it means i.e. they are difficult to disolve in water based solutions. Dimethylsulfoxid (DMSO) or ethanol act as the solvents. But the useness of these solvents is not easy, because they often have an own influence of the cells. This is the reason while the stock solutions in DMSO are diluted with water based buffers. But some

compounds are so water-insoluble that they precipitate when DMSO concentration falls below a critical value. In this case it is absolutely necessary to evaluate the solvent effect. This implicates the measurement of the effect of different concentration of the solvent on the cells.

LS174T cells were transfected with the reporter gene construct pGL-CYP3A4(-7830Δ7208-364), the expression plasmid pcDhuPXR and a mammalian ß-Galactosidase expression plasmid. The treatment of the cells took place seven hours after transfection. Figure 9 shows an overview of the effect of different concentration of DMSO. The 0.01% concentration of DMSO has no significant effect on the cells. It is comparable with the effect of a non-inducer. But a concentration of 3% greatly increases luciferase activity. These results show that it is very important to consider the effect of the solvent. To see if a compound is an inducer or a non-inducer it is necessary to substrate the relative luciferase activities which are attributed to the solvent from the relative luciferase activities which are obtained after treatment with the appropriate compound. Are the resulted values positive, it is an inducer. Are the resultes negative or incline to zero, it is a non-inducer.

Evaluation without the described compensation generate misleading results, for example to identify a compound as an inducer even though the relative luciferase activity would be attributed to the solvent.

An improved assay for screening inducers of CYP genes could be generated based on a combination of the above described steps. Advantageously, this improved assay allows effectively to eliminate sources for false positive or negative results. In contrast to the assay described in Example 5, the assay described above allows reliable identification or verification of the known inducers tested so far. In addition, the tested known non-inducers could also be verified in the improved assay. The results obtained thusfar are summarized in the following table:

Compounds	Induction	Tox. / Str ss	Solvent	Specific Induction
Inducers				
Rifampicin	+++	—	—	+
RU 486	++	+	—	+
Clotrimazol	++	+	—	+
Corticosterone	+	+	—	+
Nifedipine	++	+	—	+
Reserpine	+	+	—	+
Omeprazol	++	+	— / +	+
Pantoprazol	++	+	—	+
5β-Pregnane-3,20-dione	++	+	—	+
Phenobarbital	++	—	—	+
Non-Inducers				
Digoxin	—	+	—	—
Stauroporine	—	+	—	—
Doxorubicin	—	—	—	—

All established inducers of hepatic CYP3A4 expression induce also the CYP3A4 promoter-driven luciferase activity in LS174T (specific induction) cells as used in the improved method of the invention.

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Claims

1. A method of determinig whether a compound is an inducer of a drug metabolizing enzyme comprising the steps of:
   (a) culturing cells of the cell line LS-174T (ATCC No. CI-188) or cells derived therefrom in a nutrient medium essentially free of steroids and capable of supporting the growth of said cells, wherein said cells comprise at least one gene encoding a protein capable of regulating the expression of said drug metabolizing enzyme and a first recombinant DNA molecule which comprises a heterologous promoter operatively linked to a gene encoding a first detectable product and a second recombinant DNA molecule which comprises nucleotides –7830 to +51 or at least one repeat of nucleotides –7830 to –7209 and nucleotides –363 to +51 of the CYP3A4 gene (Accession number: AF185589) operatively linked to a gene encoding a second detectable product;
   (b) incubating said cells with a first amount of a compound or a sample comprising a plurality of compounds suspected to be an inducer under conditions which permit expression of said second and said first detectable product;
   (c) quantifying said first and said second detectable product;
   (d) determining the ratio of the amount of said second and said first detectable product; and
   (e) comparing the ratio determined in step (d) with the ratio obtained in step (d) of a corresponding assay with a control substance, wherein an increased ratio is indicative for an inducer.

2. The method of claim 1 comprising the further steps of:
   (f) culturing cells of the cell line LS-174T (ATCC No. CI-188) or cells derived therefrom in a nutrient medium essentially free of steroids and capable of supporting the growth of said cells, wherein said cells

comprise at least one gene encoding a protein capable of regulating the experession of said drug metabolizing enzyme and a first recombinant DNA molecule which comprises a heterologous promoter operatively linked to a gene encoding a first detectable product and a second recombinant DNA molecule which comprises nucleotides –7830 to +51 or at least one repeat of nucleotides –7830 to –7209 and nucleotides –363 to +51 of the CYP3A4 gene (Accession number: AF185589) operatively linked to a gene encoding a second detectable product;

(g) incubating said cells with a second amount which is increased compared to the first amount of step (b) of a compound or a sample comprising a plurality of compunds suspected to be an inducer under conditions which permit expression of said first and said second detectable product;

(h) quantifying said first and said second detectable product;

(i) determining the ratio of the amount of said second and said first detectable product; and

(j) comparing the ratio determined in step (i) with the ratio obtained in step (i) of a corresponding assay with a control substance, wherein an increased ratio is indicative for a inducer;

(k) determining the ratio of the ratios characterized in step (e);

(l) determinig the ratio of the ratios characterized in step (j); and

(m) comparing the ratio determined in step (k) with the ratio determined in step (l), wherein an increase of the ratio determined in step (l) compared to the ratio determined in step (k) is indicative for an inducer.

3. The method of claim 1 or 2, wherein said cell derived from LS-174T (ATCC No. CL-188) is obtained by introducing at least one gene encoding a protein capable of regulating the expression of a drug metabolizing enzyme and/or drug transporter gene into said cell, said gene being under the control of a promoter functional in the cell, whereby said gene is expressed.

4. The method of any one of claims 1 to 3, wherein said at least one gene regulating the expression of the drug metabolizing enzyme or drug transporter gene is an orphan nuclear receptor gene.

5. The method of claim 4, wherein said orphan nuclear receptor is the pregnane X receptor (PXR) or a variant thereof.

6. The method of any one of claims 1 to 5, wherein the said first, the said second or the said first and said second recombinant DNA molecule or gene is introduced into the cell by means of a retroviral vector.

7. The method of any one of claims 1 to 6, wherein the first detectable product is luciferase, Renilla luciferase, green fluorescent protein, chloramphenicol transferase β galactoridase, human growth hormone or alkaline phosphatase.

8. The method of any one of claim 1 to 7, wherein the second detectable product is luciferase, Renilla luciferase, green fluorescent protein, chloramphenicol transferase β galactoridase, human growth hormone or alkaline phosphatase.

9. The method of any one of claims 1 to 8, wherein said first and said second detectable products are different.

10. The method of any one of claims 1 to 9 in which the substance is a suspected agonist/activator of the activity of a CYP gene.

11. The method of any one of the claims 1 to 13, comprising the additional step of incubating said compound(s) with an S9 liver extract prior to exposing said cells to said compounds(s).

12. A method of identifying an inhibitor/antagonist to a new inducer comprising the steps of:

(a) determining whether a compound suspected to be an inducer is an inducer using the method according to any one of claims 1 to 11;

(b) repeating the method used in (a) with the additional step of treating the cells employed in said method prior, simultaneously or after incubating the cells with the inducer identified in step (a) but prior to quantifying a detectable product as specified in any one of claims 1 to 11 with a substrate suspected to be an inhibitor/antagonist to said inducer identified in step (a); and

(d) determining whether the substrate suspected to be an inhibitor/antagonist decreases or obliterates the effect of the inducer identified in step (a), wherein a decrease or obliteration is indicative for an inhibitor/antagonist.

13. A method of decreasing the toxicity of a drug, comprising the steps of:

(a) determining the type of modulation caused by said drug using the methods according to any one of claims 1 to 12; and

(b) modifying said drug to alter or eliminate the portion thereof suspected of causing said determined modulation.

14. The method of claim 13, further comprising, after step (b), the additional step of:

(c) repeating the method used to determine the type of modulation caused by said drug according to step (a) using the modified drug according to step (b).

15. A modified drug produced by the method of claim 13 or 14.

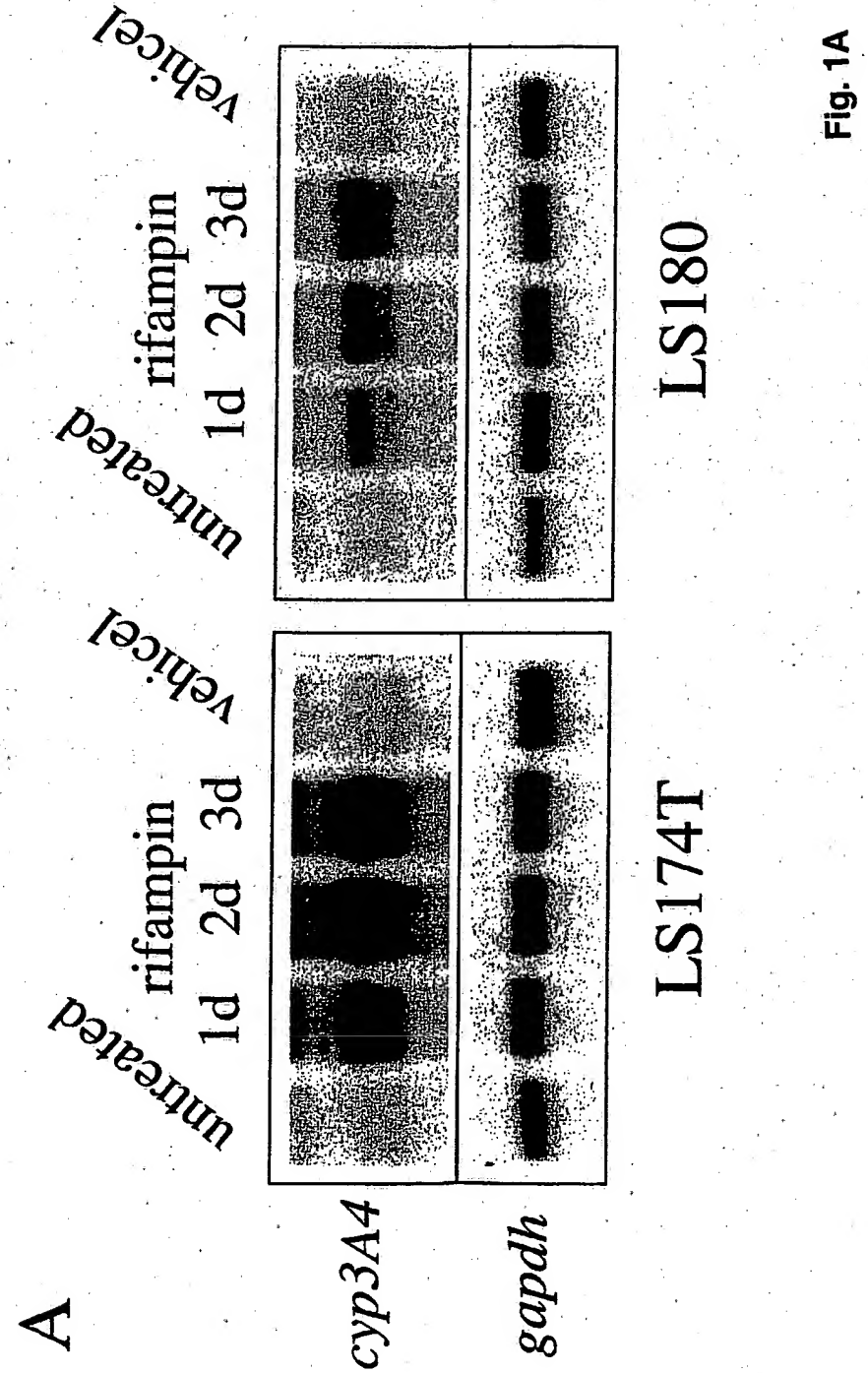
16. A method for the production of a pharmaceutical composition comprising the steps of the method of any one of claims 1 to 14 and the further step of formulating the inhibitor/antagonist or drug in a pharmaceutical acceptable carrier.

17. Use of cells from the cell lines consisting of LS-174T (ATCC No. CL-188) or cells derived therefrom or cells as defined in any one of claims 1 to 14 for the determination of whether a substance is modulator of the expression activity of a drug metabolizing enzyme or of a gene capable of regulating the expression of such a gene.

18. A test kit useful for carrying out the method of any one of claims 1 to 13, which comprises:

(a) cells of the cell line LS-174T (ATCC No. CI-188) or cells derived therefrom, in a nutrient medium essentially free of steroids and capable of supporting the growth of said cells, wherein said cells comprise at least one gene encoding a protein capable of regulating the expression of said drug metabolizing enzyme and a first recombinant DNA molecule which comprises a heterologous promoter operatively linked to a gene encoding a first detectable product and a second recombinant DNA molecule which comprises nucleotides –7830 to +51 or at least one repeat of nucleotides –7830 to –7209 and nucleotides –363 to +51 of the CYP3A4 gene (Accession number: AF185589) operatively linked to a gene encoding a second detectable product; optionally in combination with

(b) a control substance and/or means and/or instructions for carrying out the method of any one of claims 1 to 13.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LS180
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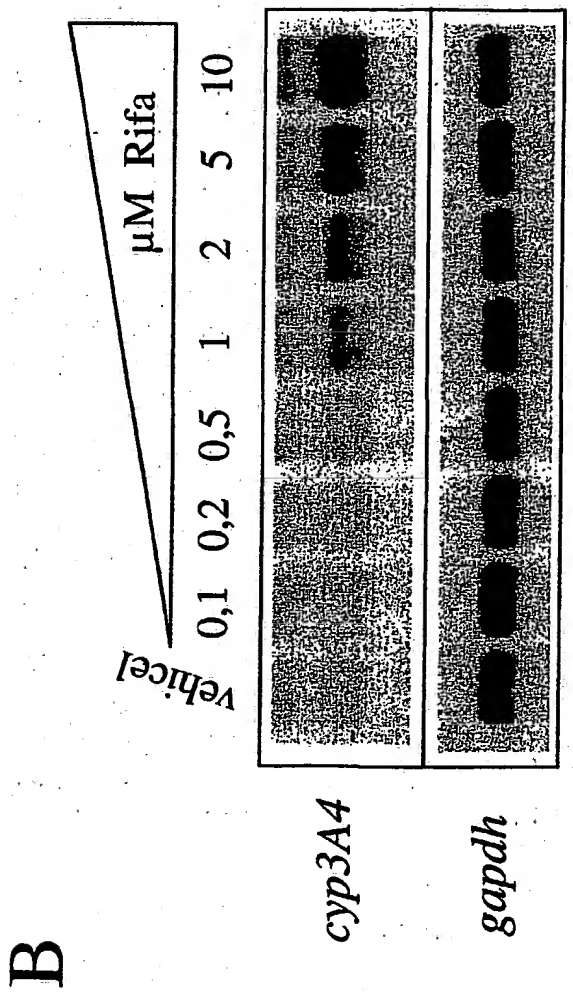
rifampin
1d 2d 3d
vehicel

Fig. 1A



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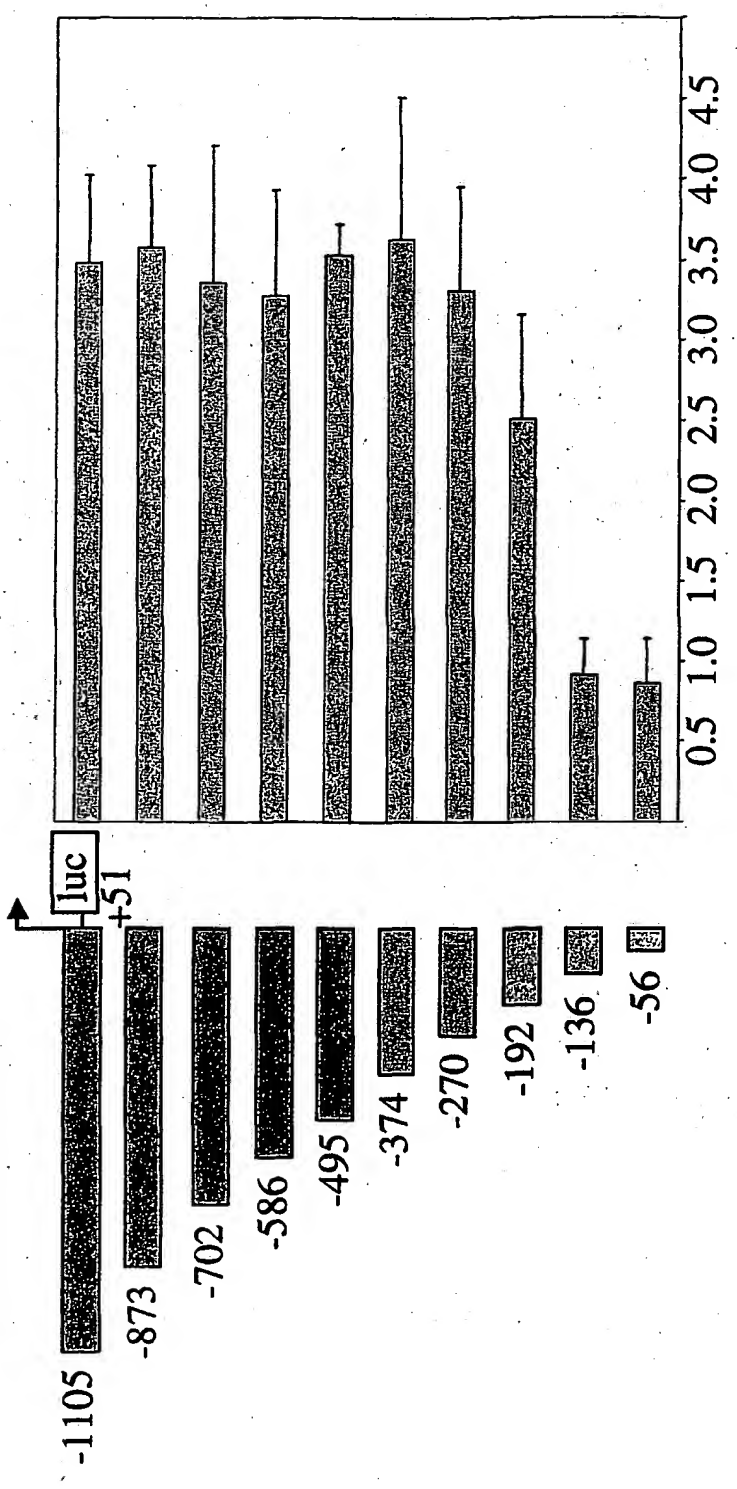
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+51
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-873
-702
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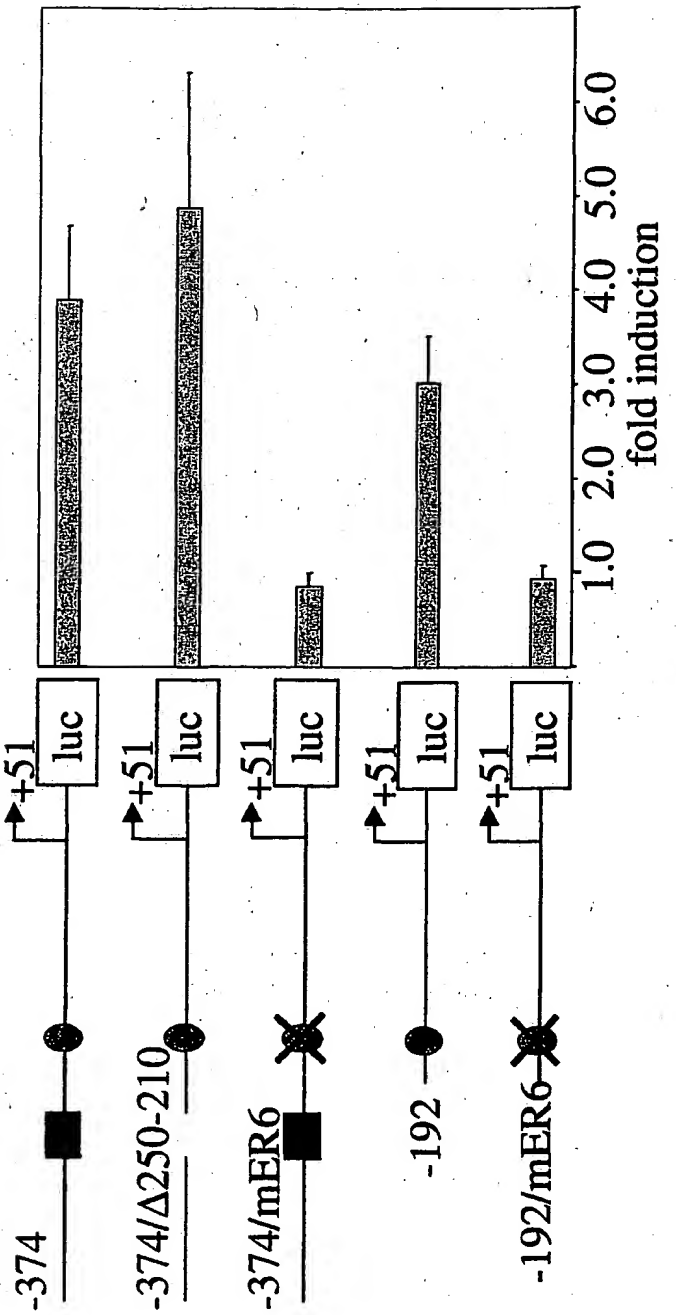
-374
-270
-192
-136
-56

Fig. 2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+51
luc
-374
-374/Δ250-210
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-192
-192/mER6

Fig. 3

A
HepG2
BN
LS180
LS174T
Caco-2 TC7
1 2 3
pxr
pxr
gapdh
B
- +
60
50
40
30
α-hPXR
C
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Fig. 4

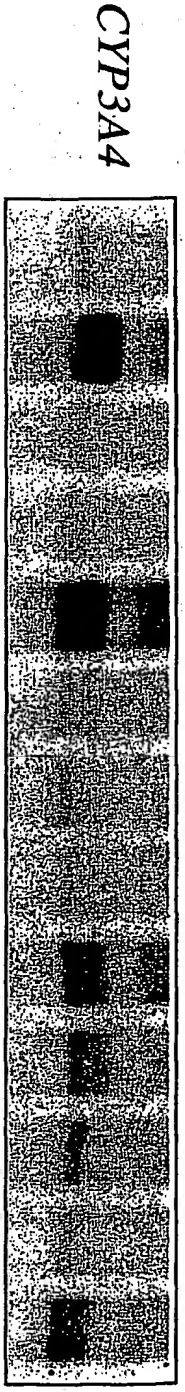
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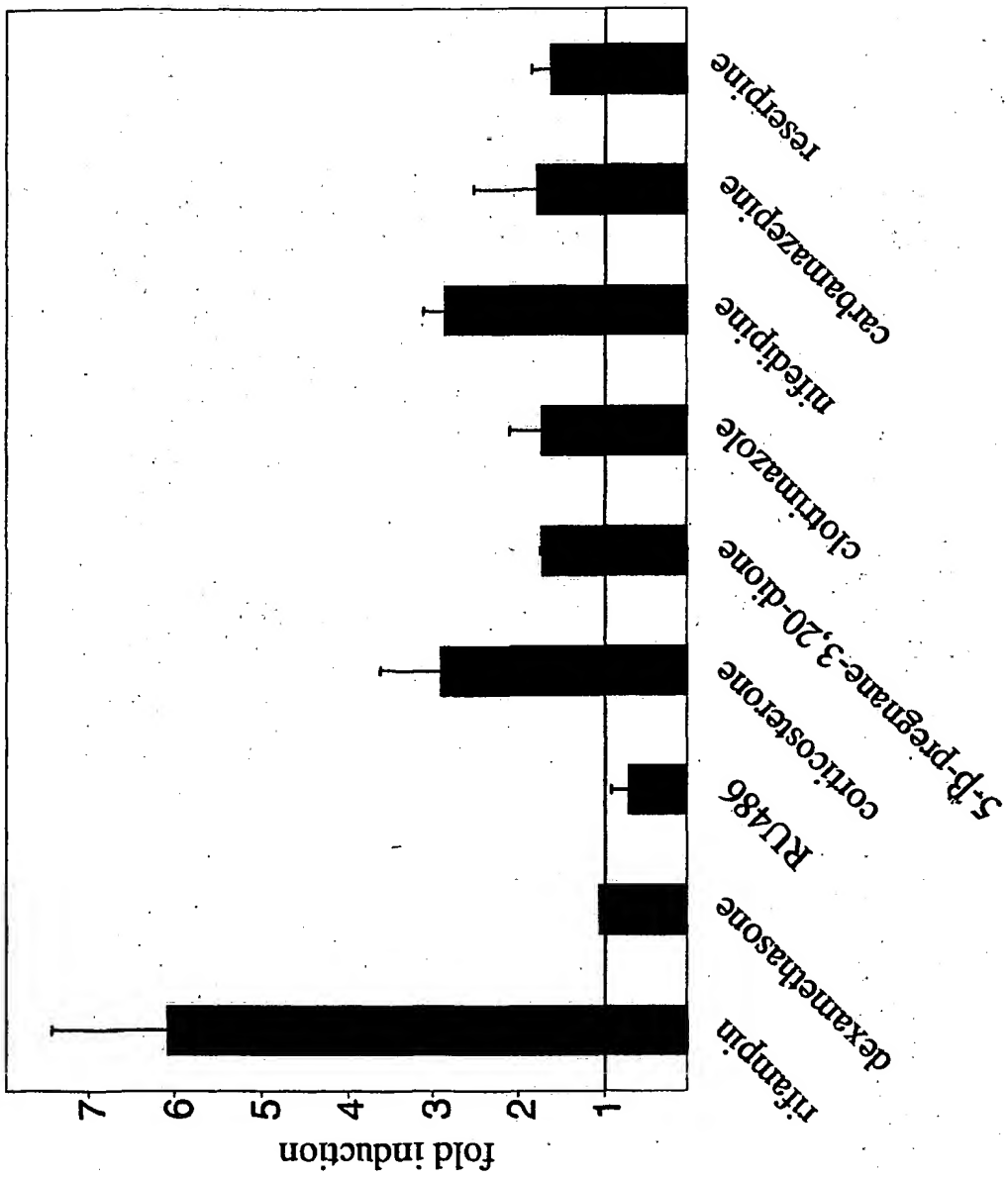
Fig. 5

Fig. 6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1
2
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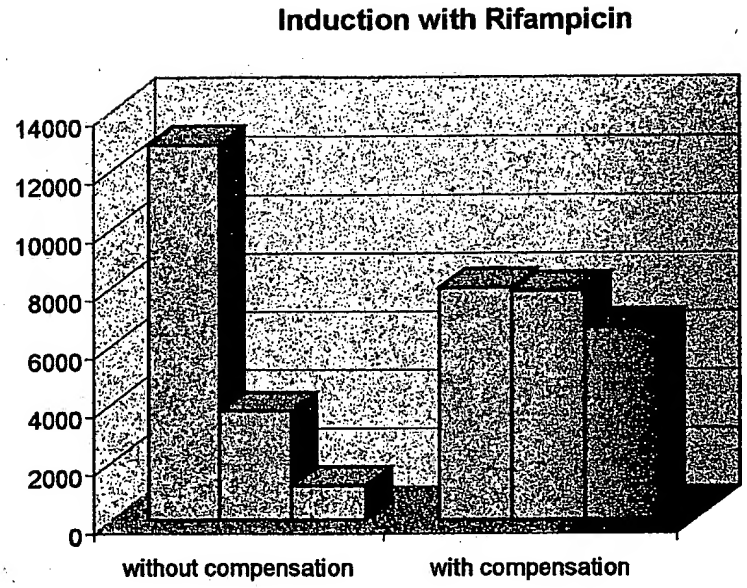
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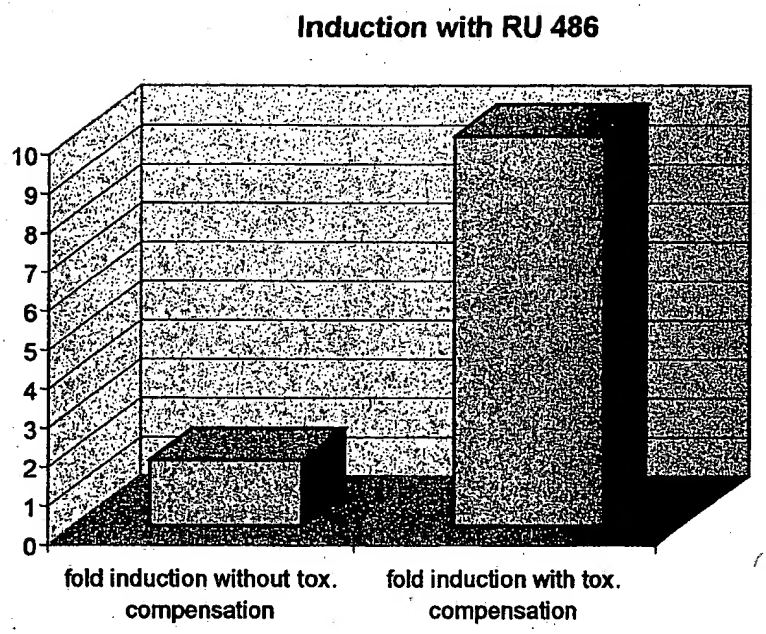
4000
2000
0
without compensation
with compensation

Fig. 7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8
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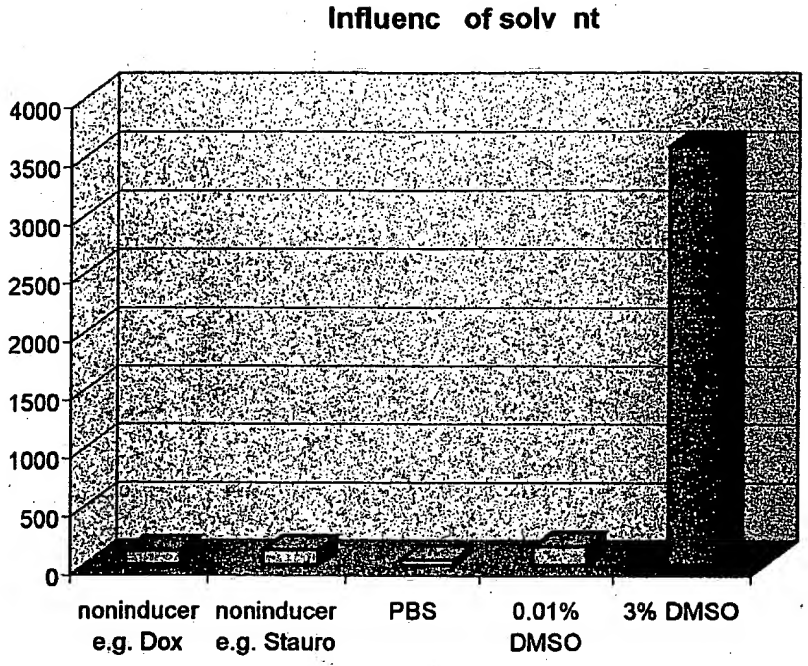
5
4
3
2
1
0
fold induction without tox. compensation
fold induction with tox. compensation

Fig. 8


Influenc of solv nt
4000
3500
3000
2500
2000
1500
1000
500
0
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noninducer e.g. Stauro
PBS
0.01% DMSO
3% DMSO

Fig. 9

SEQUENCE LISTING

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